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Cannabinoids: A Novel Treatment Strategy for Retinal Neurodegenerative Disorders

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CANNABINOIDS:

A NOVEL TREATMENT STRATEGY FOR RETINAL NEURODEGENERATIVE DISORDERS

by

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ABSTRACT

CANNABINOIDS:
A NOVEL TREATMENT STRATEGY FOR RETINAL
NEURODEGENERATIVE DISORDERS

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Eastern Virginia Medical School and
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Synthetic and naturally occurring cannabinoids are known to decrease intraocular pressure (IOP). Glaucomatous damage to the retina and optic nerve progresses even after therapy to maintain normal intraocular pressure (IOP). Topical application of cannabinoids decreases IOP while not affecting blood pressure or heart rate. Based upon their effects on other tissues, we hypothesize that these analogs reduce IOP and may also confer direct neuroprotective effects on the retina, possibly via CB1 and/or CB2 receptors. The purpose of this study is to determine if the newly synthesized CB agonists, lipid soluble O-1812 (CB 1), and water soluble O-2545 (CB 1<<2) are potent ocular anti-hypertensive and neuroprotective.

The IOP effects were tested in a rat glaucoma model, which was created Sprague-Dawley rats by ligating 3 of 4 episcleral veins. IOP increased by at least 5 mm Hg in the operated eye, measured via Goldmann tonometry under sedation. Retinal damage was induced by injecting NMDA (2 μl of 10 mM) intravitreally in Sprague-Dawley rats. In other groups O-1812 (2 mM) and O-2545 (2mM) were co-injected with NMDA. Electroretinograms (ERG) were recorded at baseline, 1 wk and 2 wk after injection.

Contralateral normal eyes served as controls. After 2 wk, retinas were flat mounted and stained with H&E.

While both O-compounds showed significant IOP decrease lipid soluble O-1812 had the most rapid onset. O-1812 was also effectively restricted a-wave amplitude loss; O-2545 was less effective. Retinal whole mounts of NMDA alone showed areas devoid of cells, while those treated with O-1812 and O-2545 were intact with an even distribution of retinal ganglionic cells. Considering both IOP and neuroprotective effects, lipid soluble O-1812 was deemed to be most effective.

Co-Directors of Advisory Committee: Dr. Peter F. Blackmore
Dr. Stephen J. Beebe

This thesis is dedicated to my parents

Dr. Suresh Samudre, MD and Jessica Samudre, RN

For their undying love and sacrifice to ensure that we had the best of everything,

for their dedication to the fields of medicine and science,

for their wisdom and understanding

for their constant support and prayers,

for being exemplary parents, role models and teachers

Thank you

...and you will know the truth, and the truth will set you free (John 8:32)

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CHAPTER I

INTRODUCTION

Elevated intraocular pressure or ocular hypertension is a major risk factor for the development of glaucoma. Reduction of intraocular pressure (IOP) retards glaucoma progression for both normotensive and ocular hypertensive patients [1, 2]. For this reason, current therapy is dominated by drugs that decrease IOP. Although drugs, such as beta adrenergic antagonists, carbonic anhydrase inhibitors, and cholinergic, alpha adrenergic or prostaglandin agonists, are currently prescribed to decrease IOP, they only indirectly alleviate glaucomatous damage. The causative factor for glaucoma related blindness is damage to the optic nerve and loss of retinal ganglion cells.

The therapeutic effect of smoking *Cannabis Sativa* on IOP was first reported in the early 19th century [3]. This plant has more than 480 chemical constituents [4]. Of these, a group of at least 66 compounds that contain only carbon, hydrogen and oxygen are known collectively as cannabinoids [5]. They represent a promising therapeutic class and have been proposed as treatment for a wide range of clinical disorders, including glaucoma. In 1971, Hepler and Frank reported that smoking marijuana lowers IOP by 25-30% in a subset of subjects [6]. Unfortunately, duration of action after smoking marijuana was relatively short, about 3-4 hours, but there appeared to be a dose-response relationship [6, 7]. Other ocular effects were conjunctival hyperemia, reduced tear production and an increase in pupil size [7]. Concomitantly there are acute systemic effects

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This dissertation follows the style of *Investigative Ophthalmology and Visual Science*

induced by smoking marijuana, such as decreased blood pressure and tachycardia [8]. In addition to decreasing IOP, cannabinoids may also have neuroprotective actions [5, 9].

A major psychoactive constituent of cannabis, delta 9 - tetrahydrocannabinol (THC), produces many of its effects through activation of cannabinoid (CB_1 and CB_2) receptors [10]. These receptors normally respond to endocannabinoids, endogenous agonists that are synthesized and released by neurons on demand. Endocannabinoids may function as neurotransmitters, neuromodulators or synaptic messengers [11]. CB_1 receptors are largely present in brain, spinal cord, and certain peripheral tissues that include lung, heart, urogenital and gastrointestinal tract and the eye [12-14]. Peripherally located CB_2 receptors are found in high concentration in cells and tissues associated with the immune system, such as the tonsils and spleen, as well as in retina [5].

Following the identification of endogenous cannabinoids and their receptors, numerous synthetic cannabinoids were synthesized. WIN-55-212-2 is the well-studied prototype for the aminoalkylindole synthetic cannabinoids that are structurally different from endogenous cannabinoids. WIN-55-212-2 activates both CB_1 and CB_2 receptors, albeit with a proclivity for the CB_2 receptors [12]. In normotensive rabbits, a single dose of either topical or systemic WIN-55-212-2 reduced IOP without apparent ocular toxicity [15], most likely through effects on CB_1 receptors [16]. Another major group of endocannabinoids are the eicosanoids, of which anandamide is a prototypic member. When released in response to insult or injury, these endogenous substances provide analgesic and neuroprotective effects. Unfortunately, anandamide is rapidly hydrolyzed

by fatty acid amide hydrolase terminating its activity. Numerous studies with structure-activity relationships have led to development of novel anandamide analogs, such as O-1812.

This dissertation focuses characterization of anandamide analog, O-1812, and THC analog, O-2545, in terms of their IOP reduction potential, neuroprotective effects and mechanism of action. Topical administration is emphasized to minimize adverse systemic and CNS effects.

CHAPTER II

BACKGROUND AND AIGNIFICANCE

Visual impairment, ranging from decreased acuity to blindness, is a major cause of morbidity, interferes with activities of daily living, and increases functional dependence, and is associated with a shorter life expectancy [17-19]. Based on the 2000 census data, nearly 0.8% Americans over the age of 40 suffers from blinding disorders with an additional 2% with low vision. While those over 80 years of age make up 7.7% of the population, it is estimated that they account for 70% of blindness; this elderly segment represents the fastest-growing portion of the population [20]. As average life spans increase, age-related loss of vision will become a growing concern.

There are a number of causes of low vision and blindness. The most common among Americans of varying ethnicities are age-related macular degeneration (AMD), cataracts, glaucoma, and diabetic retinopathy. Of these impairments, vision can be restored only in cataracts after surgical replacement with a prosthetic intraocular lens. The others AMD, glaucoma, diabetic retinopathy cause severe retinal damage and present significant obstacles in their treatment.

Etiology and Pathology of Glaucoma

Glaucoma is the second leading cause of blindness worldwide [5]. Over 66.8 million people have the condition, which has caused bilateral blindness in 6.7 million [21]. Fewer than 50% of glaucoma patients in first world countries know that they have the

disorder, and even fewer are aware of it in developing countries [22]. In the US, open-angle glaucoma is the leading cause of blindness in Hispanics with an added eight-fold increase in risk of blindness for African Americans [20]. Primary open angle glaucoma is the most common form, presenting with elevated IOP and concomitant retinal damage. Although the etiology and pathological basis of glaucoma is not yet fully understood [22], increased IOP remains a risk factor but not necessarily the cause. IOP increases when the aqueous outflow is compromised possibly due to the inhibition of the trabecular meshwork or by increased production of aqueous humor by the pars apparatus. This leads to a concordant increase in pressure across the lamina cribrosa and places stress on the axons of the retinal ganglion cells. While mechanical pressure might cause neuronal damage, increased IOP is neither necessary nor sufficient to cause neurodegeneration [23-26]. Other putative causative or contributing factors of glaucomatous neuropathy are local hypoxia due to a dysfunction in blood-flow regulation, excessive glutaminergic stimulation, oxidative stress, abnormal cellular pumps, and a compromised immune function [27]. In addition to high IOP (>21 mmHg), risk of developing open angle glaucoma is increased by age, the ratio of the horizontal and vertical diameters of the optic cup to the optic disc, family history of open angle glaucoma, and a thin central corneal measurement [28-33].

Pharmacologic Therapies for Glaucoma

It has been over a decade since a new class of pharmacologic agents was introduced for the clinical management of glaucoma [25]. Currently available therapeutic approaches for the treatment of glaucoma include prostaglandin analogs, beta-adrenergic antagonists, alpha-2 adrenergic agonists, carbonic anhydrase inhibitors and cholinergic

agonists. Each of these has a well known profile of clinical response and adverse effects. Even the first line agent for the treatment of glaucoma, timolol, as monotherapy controlled IOP in only 98 of 155 patients (63.2%) [34]. More recently, in the Ocular Hypertension Treatment Study, 40% of patients randomized to treatment required more than one medication to achieve the goal of a 20% reduction in IOP [35]. Reduction of IOP retards glaucoma progression for both normotensive and ocular hypertensive patients [1, 2]. However, the rationale for reducing IOP to manage glaucoma does not adequately address the need to curtail neuronal loss. There is currently no efficacious treatment that directly addresses glaucoma induced neuropathy.

Surgical treatment options for Glaucoma

Lowering IOP either pharmacologically or surgically is the mainstay for glaucoma management. Pharmacological approaches remain the first line therapy in most cases of glaucoma. When multiple medications are ineffective or not tolerated, surgical approaches are implemented. Argon laser trabeculoplasty (ALT) is sometimes used as initial therapy in glaucomatous eyes. In this technique, 40-50 spots with a size of 50 micron are placed radially around the trabecular meshwork with a short-term success rate of 65 – 97% [36-39]. More invasive surgical approaches include trabeculectomy, where a small portion of the trabecular meshwork is excised, and filtration surgeries, where a filtration bleb is created. As with all surgical approaches, complications due to wound healing or bleb failures are common and may require additional surgical interventions.

Cannabinoids and Glaucoma

For over thirty years cannabinoids have been touted for their potential to decrease IOP [6]. Unfortunately, cardiovascular and psychotropic adverse effects preclude systemic administration [40, 41]. Systemic administration of cannabinoids usually produces unwanted reductions in systolic and diastolic blood pressure, as well as decreased heart rate and variable changes in pupil diameter [42-44]. Moreover, the associated dysphoric psychotropic effects of these agents are documented [45].

Cannabinoids and Neuroprotection

Evidence for the neuroprotective properties of cannabinoids is based on research using mice and rat models of traumatic brain injury (TBI) [46]. In the early post-traumatic events that follow TBI, *N*-methyl-D-aspartate (NMDA) receptors are activated leading to the presynaptic accumulation of glutamate and Ca (2+). The NMDA receptor is ionotropic which allows Na+, K+ and especially Ca (2+) ions to flow in and out of cells. Over activation of the NMDA receptor by glutamate and NMDA triggers excessive Ca (2+) entry into the cells disrupting the cellular homeostatic mechanisms targeting the cell for apoptosis [47-49]. In glaucoma, the death of retinal ganglionic cells is proposed to be mediated by NMDA receptor induced excitotoxicity [50]. In response to these events, phospholipase C is activated, followed by the synthesis of diacylglycerol (DAG) and an endocannabinoid, 2-arachidonoyl glycerol (2-AG). Once 2-AG is released, it selectively activates cannabinoid receptors type 1 (CB1) to inhibit glutamate and Ca (2+) release, thereby protecting the neurons from excitotoxic damage. In a similar fashion,

anandamide, which is also an endocannabinoid produced on demand, has been demonstrated to protect rat cerebro-cortical neurons from ischemic damage. However, the endocannabinoids are hydrolyzed as rapidly as they are produced limiting the duration of effect.

Evidence for endogenous cannabinoid production in the eye is sparse, although, the presence of CB1 receptors has been demonstrated in the trabecular meshwork and ciliary process tissues [13, 51-53]. Research done in the 1970's indicated severe retinal damage when monosodium glutamate (MSG) was injected in rat skin and eyes [54, 55]. This damage was similar to that seen in glaucoma patients. Since then, several studies reported elevated glutamate levels in the vitreous humor as well as retinal Muller cells of glaucomatous eyes of humans and monkeys. Recently, the neuroprotective effect of the cannabinoid, delta 9-THC was demonstrated in a rat NMDA model of retinal neurotoxicity. In his study, El-Remessey induced retinal damage in rats by intravitreal injection of NMDA followed by intravenous administration of delta 9-THC [23]. While their neuroprotective effect was clearly established, the safety and practicality of intravenous injection of a cannabinoid is of concern. Delta 9-THC is a potent psychoactive substance eliciting the characteristic tetrad effects, namely suppression of spontaneous activity, antinociception, hypothermia and catalepsy [56]. We believe that it is possible to circumvent these systemic effects by topical application of cannabinoids. To date, no reports that establish neuroprotection as a result of topical application of cannabinoids have been published.

Brief History of Cannabinoid Research

Research on cannabinoids dates back to the 1700's, partly due to its extensive medicinal use in India and the East. Ingredients derived from the plant *Cannabis Sativa* were extracted as a resin (hashish) and were widely circulated for recreational purposes. In academic realms, a quest to identify and isolate the active components had already begun. In 1855, the Pharmaceutical society of Paris awarded Personne a prize for his extraction of a volatile oil from hemp [57]. This attempt, albeit novel, did not produce meaningful results, as this oil was inactive and contaminated with toxic terpenes [58]. Isolation of the active ingredient was complicated by the presence of numerous lipids in the mixture making the separation tedious and, in most cases, impossible. With limitations in the isolation techniques, composition of the active ingredient eluded scholars of the day. It was presumed that the active component was an alkaloid similar to that of opium [59].

In 1975, the first cannabis alkaloid was identified. Cannabisativine was isolated form a Mexican variant of *Cannabis Sativa*. However, this form was still crude, and its biological activity in man was not established owing to its low concentration in the plant itself. Meanwhile, Gaoni and Mechoulam, isolated the main psychoactive ingredient, delta 9-THC using chromatography and NMR [60]. By the late 1970's most cannabinoids were isolated and a majority of its metabolic pathways defined [59]. Cannabinoid-based drugs were developed in the mid 1980's. For example nabilone was approved for use as

an antiemetic agent in the UK and later dronabinol in France as an appetite stimulant for AIDS patients [61].

Cannabinoid Receptors

The pharmacological basis of cannabinoid action was assumed to be related to their high lipophilicity. Delta 9-THC is a potent lipophile and its biological activity considered to be similar to that of anesthetics and solvents. The isolates were racemic mixtures of delta 9-THC. Progress towards identification of a receptor-based system was hampered because of the assumption that their mechanism of action was similar to that of anesthetics rather than a receptor based system. In 1988, using tritium labeled cannabinoids, Howletts et al., identified a cannabinoid-based receptor. This discovery was an impetus for subsequently cloning the receptor [62].

Strategies to clone the receptor were based on conserved sequence motifs to clone G protein-coupled receptors. Using this concept, resultant cDNA clones from rat brain were screened against its genome resulting in identification of a CB1 receptor specific for cannabinoids [63]. This conserved sequence was screened in mouse and human genomes as well. In both rats and mice, this receptor encodes a protein made up of 473 amino acids [64]. In humans the sequence was identified to be 472 amino acids sharing a 97-99% sequence identity with rats and mice [65, 66]. The CB1 receptor is made up of seven transmembrane domains, resembling G protein coupled receptors in form and function (signal transduction). The CB1 receptor (mRNA and protein) is predominantly found in brain and neuronal tissue.

In 1993, Munro et al. cloned a new receptor from human HL-60 cells that bound delta 9-THC, aminoalkylindoles and eicosanoids. This 360 amino acid sequence was markedly different from the 472 amino acid sequence of the CB1 receptor. In fact, it shared only 48% homology with the CB1 receptor and 70-80% homology with other species. This new receptor was designated as a CB2 receptor. This receptor contains seven transmembrane domains and is predominantly found in spleen and immune tissues. Subsequently, genes for mouse and rat CB2 were cloned [67, 68]. It should be noted that the rat gene is 13 amino acids longer at the carboxyl terminal than the mouse gene.

Considerations for cannabinoid receptor agonist binding

Successful cloning of the CB receptors has spawned numerous efforts to define agonist binding sites. The CB1 receptors comprised of seven transmembrane helical domains as well as a well conserved amino terminal extracellular domain containing 117 residues [10]. However, this long amino terminal domain does not play a major role in ligand binding or recognition [69].

Successful binding of anandamide to CB1 receptor is facilitated by lysine residue (position # 192) located at the extracellular end of helix three. Substitution of this residue with alanine eliminated anandamide binding and subsequent receptor activation, but had no effect on WIN55212-2 binding [16, 70]. WIN55212-2 is the well-studied prototype for the aminoalkylindole synthetic cannabinoids that are structurally different from endogenous cannabinoids. While substitution with arginine had little effect, glutamine

eliminated any binding [71]. The corresponding lysine in CB2 receptors is located at position 109 on helix three. Substitution of this lysine with arginine or alanine had little effect on receptor binding [72].

Selectivity for agonist and antagonist binding can be localized to helices 4 and 5 for both CB receptors. For agonist binding, a residue of interest is tryptophan at the extracellular end of helix 4. Substitution with alanine or leucine eliminated binding of most known agonists including WIN55212-2. The effect of anandamide binding was not studied in this model [10]. Selectivity for antagonist binding (SR141716A, CB1 antagonist) resides at the beginning of helix 5 [68].

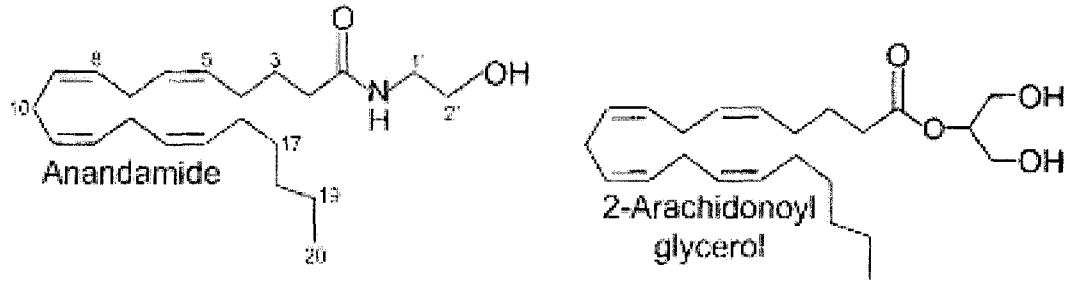

Figure 1: Structure of anandamide and 2-arachidonoyl glycerol (2-AG) [10].

Structure Activity Relationships

Anandamide is one of five endogenous cannabinoid receptor agonists found in the mammalian brain [73]. It belongs to the eicosanoid group and characterized by 20:4, n-6 series fatty acid amides (Figure 1) [10, 74, 75]. The most investigated to date have been anandamide and 2-arachidonoylglycerol (2-AG)[10].

Activity of anandamide is similar to that of delta-9-THC in that is a partial CB1 agonist with significantly less affinity for CB2 receptors [69, 76-78]. As a molecule, anandamide does not contain any chiral centers, a property that can be exploited to develop potent synthetic agents. Once released, anandamide is rapidly hydrolyzed by fatty acid amid hydrolase (FAAH) to produce arachidonic acid and ethanolamine. Martin et al. have proposed the following SAR's for developing potent synthetic CB1 agonists based on the anandamide framework [79, 80]:

1) Introduction of a methyl group on the 1 alpha carbon of anandamide resists the hydrolytic action of FAAH.
2) Substitution of the amide with a two or three carbon chain is a requirement for activity.
3) Activity is decreased if the hydroxyl is substituted. Substitution of the hydroxyl with an amino or carboxyl groups eliminates its activity.
4) Metabolic stability is increased by the introduction of a methyl in the primary carbon adjacent to the carbonyl or nitrogen.

5) Structural changes in the arachidonoyl and ethanolamide moieties yield highest potencies.
6) Activity is dramatically reduced if the chain length is decreased by two methylenes.

Development of O-Compounds

With these structure activity relationships defined, novel cannabinoid compounds, that are potent and highly selective for the CB receptors were developed by Organix Inc., (Woburn, MA), in collaboration with Dr. Billy Martin (Virginia Commonwealth University – Medical College of Virginia, Richmond, VA). Endocannabinoids like anandamide are produced in vivo in response to ocular injury but they are rapidly hydrolyzed by the enzyme FAAH. Anandamide is one of five endogenous cannabinoid receptor agonists found in the mammalian brain and also the most investigated to date, along with 2-arachidonoylglycerol (2-AG). Activity of anandamide is similar to that of delta-9-THC in that is a partial CB1 agonist with significantly less affinity for CB2 receptors [69, 76-78]. As a molecule, anandamide does not contain any chiral centers, a property that can be exploited to develop potent synthetic agents. Once released, anandamide is rapidly hydrolyzed by fatty acid amid hydrolase (FAAH) to produce arachidonic acid and ethanolamine. The newly synthesized series of O-compounds include O-1812 and O-2545. Compared with anandamide, O-1812 has higher affinity for the CB1 receptor. The methyl substituent in the 1 alpha position reduces its susceptibility to hydrolysis by FAAH [79]. Even though O-2545 is a nonselective with respect to CB1/CB2 receptors, it has the advantage of being water-soluble.

Utilizing a metabolically stable anandamide analog, (R)-1'-methyl-2'-hydroxy-ethyl-arachidonamide (Met-anandamide), a 1,1'-dimethylpentyl-Met-anandamide construct was created and a cyano group was added to the C-20 atom to create O-1812 (Figure 2) [79]. By incorporating a methyl in the 1' carbon atom, O-1812 is resistant to enzymatic degradation by FAAH. A cyano substitution gives O-1812 a 500 – 1000 fold increased selectivity for the CB1 receptors over CB2, which is greater than the native anandamide [79]. Anandamide-like cannabinoids, such as O-1812, resist the development of tolerance, unlike delta 9-THC, suggesting a unique pharmacology that only partially overlaps with traditional cannabinoids [81]. O-1812 is also implicated in seizure modulation as it was determined to be a potent anticonvulsant [82].

High lipophilicity associated with delta 9-THC, as well as other synthetic cannabinoids, is a constraint on solubility of these drugs. Cannabinoids require either surfactant or a water miscible substance, like albumin or Tween 80, to solubilize [80, 83]. A common vehicle used is a mixture of ethanol, Emulphor and saline, but even when dissolved in this vehicle, the cannabinoids will precipitate [84, 85]. It is impractical to use these vehicles in a chronic application scenario as the vehicle itself may contribute to adverse effects, for example chronic application of ethanol can lead to corneal debridement when applied topically. To avoid these pitfalls, numerous attempts to create water-soluble cannabinoids have been made.

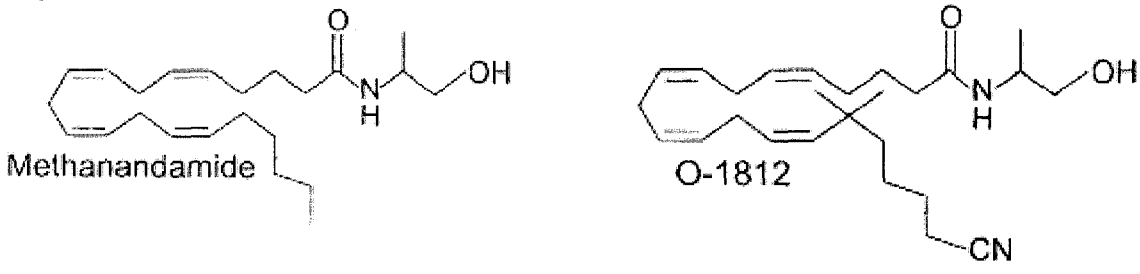

Figure 2: Structures of O-1812 and methanandamide [10, 79].

Zitko was successful in his attempt to prepare a water soluble cannabinoid [86]. Delta 9-THC was converted to a morpholinobutyryl ester and conjugated with hydrochloride to make it water-soluble. Like its parent compound, this esterified compound retained its pharmacological activity. More recently, Martin et al. have developed cannabinoid compounds that are considerably potent than delta 9-THC, more specifically, O-2545 [87]. Commercially available 5-cyano-dimethoxyresorcinol was a starting ingredient which was converted to a t-butyl dimethylsiloxy derivative and treated with an imidazole to produce O-2545 [87, 88]. Substitution with imidazole readily forms hydrochloride salts making the analogs water soluble and does not require metabolic conversion to an active molecule like ester analogs [87]. This substitution also imparts a high CB1 affinity but even a higher CB2 receptor affinity. When dissolved in saline, O-2545 was 40 fold more potent that delta 9-THC and was 20 times less lipophilic than delta 9-THC [87].

CHAPTER III

HYPOTHESIS AND SPECIFIC AIMS

Glaucoma is a group of diseases that cause retinal and optic nerve damage. Although increased IOP is a contributing factor for glaucomatous damage, many people develop this damage with normotensive IOP. Although numerous pharmacologic agents are available to control elevated IOP, none directly address retinal and optic nerve neuropathy. This dissertation focuses characterization of the anandamide analog, O-1812, and THC analog, O-2545, in terms of their effect on IOP, neuroprotective effects and the mechanism of action responsible for these effects. Topical administration is emphasized to minimize adverse systemic and CNS effects.

Preliminary studies/progress report

These studies are designed based upon the suggestion by Green that endocannabinoids may have neuroprotective effects [42]. We employed a novel technique, the electroretinogram (ERG), to provide in vivo evidence of neuroprotection in a rat NMDA model that mimics glaucomatous damage.

Functional analysis of the retina through ERG:

In diseases, like glaucoma, where the retinal integrity is compromised, ERG is an effective tool to assess the onset, severity and quantitate the loss of functional photoreceptors [89]. By documenting the retinal response to light stimulation, this method

can accurately measure the functionality of photoreceptors and supporting retinal cells, such as amacrine cells. ERG records the electrical response to light stimulus in dark-adapted eyes. Electrical responses, indicative of retinal function, are quantified and recorded electronically resulting in a waveform that is composed of a-waves, b-waves and oscillatory potentials (Figures 3). The a-wave represents activation of the rods and the b-wave the cones.

Our preliminary ERG studies demonstrate, a diminished amplitude and corresponding area of both a- and b-waves after 2 wks of NMDA treatment, synonymous with glaucomatous damage (Figure 3), which are similar to those reported by Fortune et al. [89].

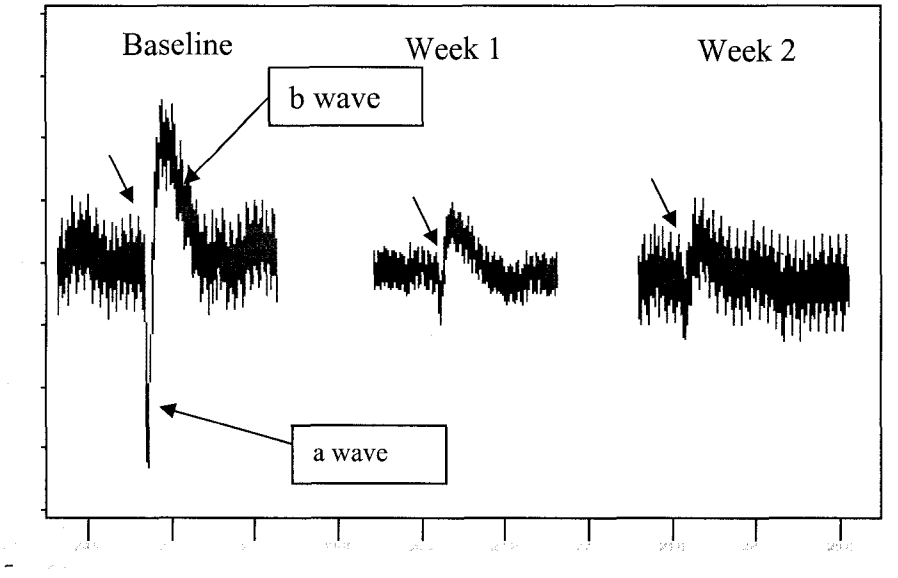

Figure 3: ERG a- and b-waves 2 weeks after NMDA injection.
Animals were dark adapted and stimulated with brief bright light flashes.
Arrows indicate light stimulation.

Chronic treatment with synthetic cannabinoids:

Our preliminary experiments suggest that over an extended period in an ocular hypertensive model, topically applied WIN 55212-2 is an effective and non-toxic ocular hypotensive agent [90]. Rats were randomly assigned to either the WIN 55212-2 (Tocris, Bristol, UK) or vehicle treated group. For 4 weeks the right eye of 5 rats was treated topically three times per day between 8 AM to 6 PM, with 20 μl of WIN 55212-2 (0.5 %). As a control using the same dosing regimen, 4 other rats were treated with Tocrisolve™ alone for 4 weeks (vehicle treated group). IOP, heart rate, and blood pressure were measured weekly, between the first and second daily doses. At the end of 4 weeks, there was a 1-week washout period to determine if IOP returned to baseline. Rats were allowed to recover without drug or vehicle treatment for 1 week before the final measurements on week 5.

Baseline IOP was 14.1 ± 0.6 mmHg in the operated eye; BP = 134±8/98±8 mm Hg; HR = 349±30 BPM. IOP decreased rapidly in the WIN-55-212-2 treated group. After one week of treatment IOP was 70% of baseline. IOP remained significantly decreased during the entire topical WIN-55-212-2 treatment period. The maximum decrease in IOP was seen after 3 weeks of treatment when IOP decreased to 47% of baseline. IOP returned to baseline (week 5) after the drug was discontinued (week 4) [91, 92]. The vehicle, Tocrisolve™, alone had no effect on IOP. The cornea, conjunctiva and anterior chamber were normal with no signs of ocular inflammation. There were no effects on HR or BP, such as those associated with systemic administration. A more

comprehensive report of WIN 55-212-2 effect on IOP after acute and chronic administration is included in chapter 9.

Experimental design

Specific aim #1: *When applied topically, how effectively do cannabinoids penetrate the ocular structures?*

A relatively new method for the elucidation of pharmacokinetic profiles in vivo is microdialysis. It has been successfully utilized for regional sampling of low-volume compartments of many tissues, such as the brain, joints, kidney, and eye [93]. By removing a minute amount of aqueous humor and re-perfusing an equivalent volume, microdialysis sampling allows for serial measurements of the aqueous fluid in order to create a complete concentration versus time curve for an individual subject. Rittenhouse et al used microdialysis sampling to produce intra- and inter-animal absorption profiles of propranolol after topical and intracameral injection, showing it to be an accurate and easily reproducible method [93]. In this study, we will use a similar method to examine the pharmacokinetics of lipophilic and hydrophilic O-compounds after topical ocular administration in an artificially perfused rat model.

For artificial perfusion, Sprague Dawley rats will be first euthanized, a Y incision will be made to expose chest cavity. A cannula will be placed in the descending aorta and all systemic circulation will be blocked except for the internal carotid arteries.

Perfusion with PBS will be adjusted to maintain a carotid pressure of 90 mmHg. Dialysis probes will be placed in the anterior and posterior chamber of the perfused animal. The flow rate for the probes will be 1 μl/min and dialysate samples will be collected every 10 min. Cannabinoid concentration will be quantified by HPLC, using a modification of the method described by Barberi-Heyob and colleagues, with a 5-μm reverse-phase column (Prodigy 5 μm ODS(2) analytical column, 150 x 4.6 mm, Phenomenex, Torrance, CA) at ambient temperature with a mobile phase of acidified acetonitrile water (pH = 4.0) solution [94, 95]. The flow rate will be 1 mL/min with UV detection (254 nm). Chromatograms will be analyzed using Beckman System Gold software (Beckman Instruments, Inc., Fullerton, CA).

Specific aim #2: *Do endocannabinoid analogues reduce IOP? What is the preferred receptor type for this effect?*

Hypothesis: In previous experiments with WIN 55212-2 1%, IOP decreased by 30%. WIN 55212-2 is a mixed CB agonist. Because O-compounds are also CB agonists, we hypothesize that the O-compounds will be as efficacious as WIN 55212-2 in reducing IOP.

Null hypothesis: If there is no decrease in IOP, then either the O-compounds are not efficacious ocular CB agonists or they are subject to rapid degradation i.e. they do not reach their intended targets at therapeutic concentrations. If this is the case then other

strategies of drug administration and synthesis of more potent analogs need to be considered.

To determine the receptor that is responsible for the IOP effect, selective CB1 antagonist, e.g. SR141716A, or a selective CB2 antagonist, e.g. SR144528, will be used to block the ocular effects of the agonist. To establish the mechanism of action including correlation between efficacy and effects on CB1 and CB2 receptors, other rats will be retreated with either a CB1 or CB2 receptor antagonist before topical treatment with the nonspecific receptor agonists as described above.

Specific aim #3: *Do endocannabinoid analogues confer neuroprotection as reflected by changes in the ERG? Which receptor (CB1 or CB2) participates in the neuroprotective effect?*

Hypothesis: In the amygdala, CB agonists O-1812 and O-2545 are neuroprotective. In the CNS or in neural tissues, they decrease oxidative damage and also increase neuroglial proliferation [56, 81]. The retinal ganglionic cells are of glial origin and, therefore, we hypothesize that the retinal ganglionic cells would show similar profiles. However, in the ocular environment proliferation of the retinal ganglionic cells is not possible due to physiological constraints. Thus, only the neuroprotective effect of these CB agonists will be evaluated in our NMDA model.

Table 1: Experimental Plan

Treatment Group	Pretreatment	NMDA
Untreated Control		
Positive Control		X
O-1812	X	X
O-2545	X	X

Null Hypothesis: If these drugs do not show functional neuroprotection, then the retinal ganglionic cells have an environmental constraint that restricts them from responding like their progenitors. Other strategies, such as cell culture experiments, will be needed to determine if, indeed, the ocular environment is the constraint.

Rats will be randomly assigned to one of 4 treatment groups (Table 1). Some rats will be pretreated with topical application of either O-1812, a lipid soluble CB analog, or O-2545, a water soluble CB analog. In our prior experiments with glaucomatous rats, both significantly decreased IOP. For the two treatment groups, each rat will receive 3 doses at 2 hour intervals before injection of NMDA. Both the untreated control and positive control will receive phosphate buffered saline (PBS) using the same treatment regimen.

The primary criterion for assessing neuroprotective effects of CB analogs includes a significant preservation of the ERG waves. ERG measurements will be performed at baseline and at 1 and 2 weeks following administration of NMDA. Rats will be sacrificed after 2 weeks for morphological analysis of the retina including retinal ganglionic cell counts. Groups treated with CB analogs should have a decrease in IOP without systemic effects, e.g. stable heart rate and blood pressure.

In the amygdala, O-1812 and O-2545 have exhibited neuroprotective effects through activation of CB receptors [96]. In the eye, in addition to the CB1 receptor, a CB2 receptor is also implicated. If neuroprotection is observed in the eye, then we will

determine which receptor (CB1 or CB2) is activated to elucidate the mechanism of neuroprotection.

These experiments will determine whether the effects of each efficacious cannabinoid are mediated via CB receptors. Receptor activity will be inferred based on the ability of either a selective CB1 antagonist, e.g. SR141716A, or a selective CB2 antagonist, e.g. SR144528, to block the ocular effects of the agonist. To establish the mechanism of action including correlation between efficacy and effects on CB1 and CB2 receptors, other rats will be retreated with either a CB1 or CB2 receptor antagonist before topical treatment with the nonspecific receptor agonists as described above. If neuroprotection is not mediated via the CB receptor system, then other likely areas of interest would be to study the antioxidative effects of these drugs.

In addition to the activation of CB receptors, the mechanism of action needs to be further elucidated by studying Ca [2+] influx into the retinal ganglionic cells. When NMDA binds to its receptor, it triggers Ca [2+] entry into cell. This produces increased calcium binding to the intracellular ryanodine receptor-coupled Ca [2+] channel and subsequent increase in Ca [2+] released from the endoplasmic reticulum (ER). Increased Ca [2+] will eventually lead to apoptosis (Figure 4). Hypothetically, CB agonists reduce intracellular Ca [2+], thus mitigating the effects of increased Ca [2+] caused by NMDA. This effect is mediated via CB1/G-protein coupled inhibition of adenylyl cyclase. In this preliminary study, the intracellular Ca [2+] will be monitored after treatment with NMDA only or in the presence of O-1812 or O-2545.

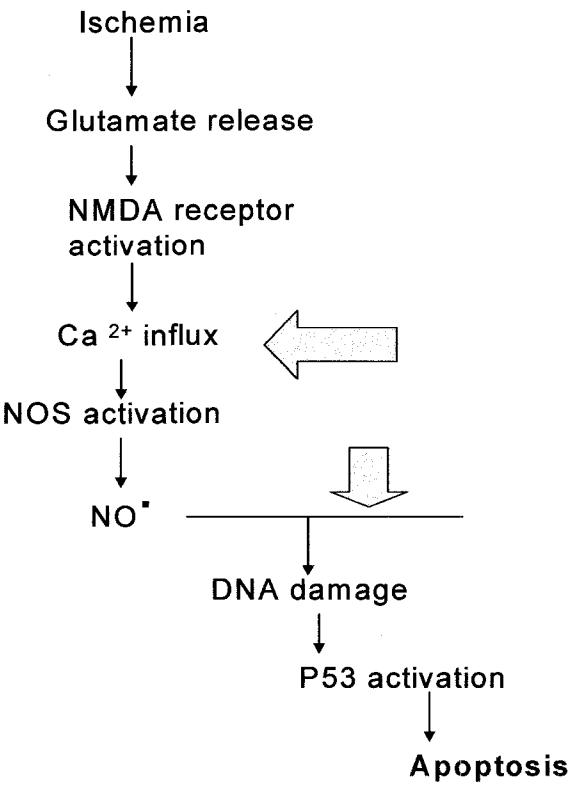

Figure 4: Proposed pathway leading to retinal ganglionic cell loss due to apoptosis. Arrows indicate site of action for cannabinoids.

Specific aim #4: *Because they have a different mechanism of actions, is there an additive effect on IOP by combining O-compounds with currently available glaucoma medications?*

As described in the introduction, currently available therapeutic approaches for the treatment of glaucoma focus mainly on the reduction of IOP. However, up to 50% of patients cannot be maintained on single drug therapy; most require use of two or even three drugs to control their IOP [97]. Commonly used classes of agents are β-adrenergic antagonists (timolol), α-adrenergic agonists (brimonidine), prostaglandins (travoprost) and carbonic anhydrase inhibitors (dorzolamide). Timolol lowers IOP by decreasing aqueous humor production while brimonidine, in addition to decreasing aqueous humor production, has the added benefit of increasing uveoscleral outflow [98, 99]. Dorzolamide reduces aqueous humor formation while prostaglandins increases uveoscleral outflow [100, 101]. We hypothesize that O-compounds are more efficacious than the currently available agents.

The primary purpose of this study is to compare efficacy of timolol, dorzolamide or brimonidine as monotherapy or in combination with endocannabinoid analogs, O-1812 or O-2545, in a rat glaucoma model. All drugs will be administered individually and IOP measured using a Tonolab™, a new instrument specifically designed to measure IOP in rats and mice. For combination therapy, timolol, dorzolamide or brimonidine will be administered first followed by O-1812 or O-2545. For all conditions, IOP will be measured 30, 60 and 120 min after drug administration.

CHAPTER IV

METHODS AND MATERIALS

Animal Subjects

All studies were conducted in accordance with the Principles of Laboratory Animal Care (NIH Publication No. 85-23, revised 1985), the OPRR Public Health Service Policy on the Humane Care and Use of Laboratory Animals (revised 1986), the U.S. Animal Welfare Act, as amended, as well as the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Protocols were approved by the Eastern Virginia Medical School (EVMS) Institutional Animal Care and Use Committee.

Rat Ocular Hypertensive Model

In human glaucoma, a gradual progression of IOP leads to various retinal deficits. This condition can be mimicked in an animal model by ligation of major outflow vessels. To elevate IOP, male rats weighing ~ 200 gm (14 Sprague Dawley, 4 Brown Norway, 5 Long Evans and 5 Wistar) underwent vortex vein ligation (Harlan, Indianapolis, IN). Surgeries were performed on the right eye (OD) while the contralateral left eye (OS) served as a normal non-operated control. A detailed explanation of the surgical technique is described in chapter 5, the development of a rat ocular hypertensive model.

Rat N-methyl-D-aspartate (NMDA) model

As a model to induce reproducible damage to the photoreceptors and retinal ganglionic cells, male Sprague Dawley rats weighing ~ 200 gm were injected intravitreally with 2 μl of 10 mM NMDA. All injections were made in the right eye (OD) while the contralateral left eye (OS) served as a normal non-modified control. In these experiments only damage to the photoreceptors was measured as a function of ERG a-wave amplitude. Although retinal damage was evident within 24 hours of NMDA injection, only 2 week measurements were reproducible.

Measurement of IOP

IOP response is a primary outcome measure for drug efficacy. Although IOP can be measured with a variety of instruments, accurate measurements can only be obtained by applanation or direct rebound tonometry. In this dissertation, Goldmann applanation tonometry and an electromagnetic piston rebound Tonolab® will be used to directly measure IOP.

Goldman applanation tonometer:

IOP measurements were performed under mild sedation (ketamine 40 mg/kg and acepromazine 6 mg/kg) with a custom machined Goldmann applanation tonometer. Two readings per eye were taken at each time interval, and averaged. Although Cohan and Bohr reported Goldmann applanation in the conscious Brown Norway rat, other

strains were not as docile [102]. We found that firm restraint of the conscious rats increased variability. To reduce the likelihood of erroneously elevated IOP readings secondary to the Valsalva maneuver, mild sedation was used.

Tonolab®:

In chapter 9, IOP measurements were made with a Tonolab® (Colonial Medical Supply Co. Inc., Franconia, NH). To reduce the likelihood of erroneously elevated IOP readings secondary to the Valsalva maneuver, mild sedation was used (ketamine 40 mg/kg and acepromazine 6 mg/kg).

Electroretinogram (ERG)

ERG is a non-invasive measure of retinal function. Damage to the different layers of the retina can be assessed by quantitating the amplitude of the a-waves (photoreceptors and bipolar cells) and b-waves (inner retinal function). This test can be performed photopically (light adapted) or scotopically (dark adapted). Maximal retinal response can only be obtained scotopically. In this dissertation, scotopic ERG was used to determine retinal function for the ligated ocular hypertensive model as well as experiments with NMDA model. Scotopic ERG changes were measured only at terminal points (32 - 72 weeks) in Sprague Dawley rats with sustained IOP elevation (n=10) to determine the extent of retinal damage. For the NMDA model, ERG's were obtained at 1 and 2 weeks after NMDA injection. NMDA was also used as a positive control in other experiments, which reduces the amplitude of both a- and b-waves [89].

ERG Procedure:

Rats were dark adapted for at least 4 hr. Eyes were dilated with atropine (1%, Falcon Pharmaceuticals, Fort Worth, TX). To restrict rat mobility, they were anesthetized with acepromazine (12 mg/kg, IP) and ketamine (80 mg/kg, IP) followed by topical proparacaine (0.5%) and methylcellulose gel (GPS 2.5%, Wilson Ophthalmic Corp, Mustang, OK). Custom made AgCl electrodes (Warner Instruments, Hamden, CT) were placed on the apex of the cornea. Stimuli consisted of 10-μsec flashes of unattenuated white light (30 cd/mm) generated by a Ganzfeld bowl photo stimulator (Grass Instruments, PS22, Quincy, MA). ERG's were recorded separately in each eye, while the contralateral eye was covered with an eye patch. ERG responses were differentially amplified (1-1000 Hz) with a driver amplifier (Grass Instruments, Model 7DAF Polygraph, Quincy, MA). Data were acquired digitally via DASYLab® (Bedford, NH). The contralateral normal eye served as an age matched negative control, e.g. response in a normal, undamaged eye. Differences in amplitude of the a- and b-waves between the operated eye and the contralateral eye were calculated and analyzed.

Fundus Examination

In glaucoma, severe degeneration of the retina and optic nerve contribute to an increase in the size of the optic cup relative to the optic disc. A fundus examination can detect changes in the optic cup and disc. With electronic documentation of the fundus, accurate cup to disc ratios to estimate the severity of glaucoma can be obtained. Glaucomatous changes in the fundus were observed by slit-lamp examination on

anesthetized rats. Retinal images were recorded with a 3.2 mega pixel digital camera (PowerShot S1 IS, Cannon, Tokyo, Japan) retrofitted to a slit lamp (Zeiss, Germany). Full resolution images were obtained with a constant optical zoom (10 X) and processed with Metamorph™ image analysis software. Cup to disc ratios were obtained for area, height, and width using Metamorph™ image analysis. For area measurements, the outer edge of the cup and disc were selected, pixels were counted, and the cup to disc ratio was determined. For individual height and width cup to disc measurements, diameters for each was obtained through a central plane. The ratios were then expressed as the cup diameter to the outer disc diameter.

Assessment for ocular irritation

Assessment of ocular irritation is possible by careful observation of anterior segment structures with the use of a slit lamp. Each structure of the anterior segment is evaluated for signs of inflammation and quantitated using a semi-quantitative scale as developed by MacDonald and Shadduck [103]. In our experiments, slit lamp examination (HAAG-STREIT, Bern, Switzerland) was performed by an independent knowledgeable observer using a modified MacDonald-Shadduck Semi-quantitative Scale for Ocular Irritation [104]. The exam was conducted at the beginning and end of each experiment to determine if the topical application of cannabinoids or vehicle caused ocular irritation. Each eye was assessed for signs of inflammation, conjunctival chemosis/swelling, conjunctival discharge, aqueous fibrin/flare, loss of the corneal light reflex, obscuration of iris structures, and corneal opacity/vascularization/staining. They were rated on a 4 point scale where 0 represented normal.

Confocal Examination

In the past, invasive histological techniques such as scanning electron microscopy were necessary to obtain detailed corneal (epithelium and endothelium) morphology. As with any histological technique the tissue has to be excised and longitudinal measurements are not possible. With confocal microscopy, the cornea can be optically sectioned making it possible to visualize and electronically document the corneal epithelium, stroma and endothelium. In addition, this method is non-invasive making it possible to obtain multiple measurements on the same subject during the study period. In this dissertation, repeated noninvasive in vivo visualization of cornea and anterior segment for the presence, location and number of inflammatory cells, as well as fibrin, hyper-refractile bodies and changes in stromal and endothelial cell morphology was performed by confocal microscopy (ASL 1000, Advanced Scanning Limited, New Orleans, LA), documented using a CCD camera (Kappa Opto-electronics Inc, Monrovia, CA) and recorded with SVO-9500MD VCR (Sony Corporation, Tokyo, Japan). Prior to applanation, rabbits were sedated with acepromazine, 2.5 mg/kg, and ketamine, 25 mg/kg, plus topical administration of 0.5% proparacaine. A surface scan was performed to verify proper applanation. Corneal thickness, epithelium, stroma, endothelium and aqueous chamber were optically sectioned in at least 3 representative areas. Endothelial cell shape changes, infiltration of inflammatory cells into the corneal stroma and anterior chamber were documented. At least 4-6 trans-corneal scans were collected for analysis using the Metamorph® imaging system (Universal Imaging, Downingtown, PA). Three readings were averaged to calculate corneal thickness.

Heart rate and blood pressure

Heart rate and blood pressure were used to evaluate potential systemic effects of topically applied CB agonists and adrenergic antagonists. A standard tail cuff apparatus was used to measure heart rate (HR) and blood pressure (BP) on sedated rats. A pulse amplifier (Model 29, IITC, Woodland Hills, CA) was placed in line for digital data conversion. Data were processed with DasyLab® generated analysis module (Version 6.0, Dasytech, Amherst, NH).

Histology

Histologic examination accurately documents any morphologic changes during the study period. In this dissertation, histology was performed on retinal flat mounts and retinal cross sections to obtain RGC counts as an adjunct measure of neuroprotection.

Histology for retinal flat mounts:

Eyes were enucleated and fixed in 10% phosphate buffered formalin (Fisher Scientific, Fair Lawn, NJ) for at least 1 wk. For retinal whole mounts, retinas were harvested by bisecting the eyes peri-limbally and removing the anterior segment. The remaining posterior segment cup was flattened with 4 radial cuts after which the retinal layer was carefully dissected from the underlying choroid and sclera. The flattened retina was placed vitreal-side up on glass slides (Fisherbrand Superfrost, Pittsburg, PA), embedded in Aqua-Mount® (Lerner Laboratories, Pittsburg, PA), and stained with 10 X dilutions of Hematoxylin and Eosin. For positive identification of RGC, retinal flat

mounts were compared with cross-sections for cell shape, placement and distribution. All slides were digitally photographed (Opelco) and quantitatively analyzed with Sigma Scan (Systat Software Inc., Richmond, CA). Cell counts were performed by knowledgeable masked observers. For each retina, at least 2 microscopic fields of 50 μm^2 were captured per quadrant at predetermined distance from the optic cup (200 um). The total retinal area was approximately 500 μm^2. RGC counts were averaged for each field and comparisons made between operated, contralateral control and positive control NMDA treated eyes. Retinal cell counts are expressed as cells per 50 $\mu m^2 \pm SD$.

Histology for retinal cross-sections:

Operated and contralateral control eyes were enucleated and fixed in 10% phosphate buffered formalin (Fisher Scientific, Fair Lawn, NJ) for at least 1 week. For retinal cross sections, whole eyes were embedded in cryo media (Tissue-Tek® O.C.T. Compound, Sakura, Torrance, CA). Sections of 40 um thickness, at the level of the optic nerve head, were cut and mounted on superfrost glass slides (Fisherbrand Superfrost, Pittsburg, PA), and stained with hematoxylin and eosin. The slides were digitally photographed at 20X magnification. Retinal ganglionic cell counts were performed on 200 um sections of the retina, for a total retinal length of approximately 12 mm documented at 20X magnification. Retinal cell counts are expressed as cells per 200 um ± SD. All quantitations were performed with SigmaScan® (Systat Software Inc., San Jose, CA)

RGC-5 Cells

RGC-5 Cells were obtained from Dr. Neeraj Agarwal, University of North Texas Health Science Center, Fort Worth, TX. They are retinal ganglionic cells isolated from Sprague Dawley rat retinas and then transformed into a ψ2 E1A viral vector. The transformed RGC-5 cells share similar characteristics with native RGC cells in that they express Thy-1 and Brn-3C and are sensitive to glutamate excitotoxicity and neurotrophin withdrawal [105].

Ca [2+] Measurements

Glutamate excitotoxicity induced by NMDA causes cells to mobilize Ca [2+] and initiate the apoptosis pathway. It is hypothesized that cannabinoids prevent the mobilization of Ca [2+] thereby preventing cellular apoptosis. Measuring Ca [2+] influx in the retinal cells is therefore necessary to understand the mechanism of action of the cannabinoids. RGC-5 cells were plated on a 96 well plate (BD Falcon, Franklin Lakes, NJ) until confluent. All cells were then treated with NMDA (1mM). After NMDA treatment, some cells were incubated with 10 uM of either O-1812 or O-2545 for 1 hr. Intracellular calcium was measured using fura-2/AM (Molecular Probes, Eugene, OR). Cells were loaded with fura-2 (2 mM) at the beginning of the experiment and Ca [2+] measurements were made at baseline, 30 and 60 min after drug treatment using a SpectraMAX™ Gemini (Sunnywale, CA) spectrofluorometer (excitation wavelength of 340 nm and 380 nm and an emission wavelength of 505 nm). At the end of the

experiment, all cells were incubated with, A23187 (Calcimycin, Cole Parmer, Vernon Hills, IL) a calcium ionophore, after which a final Ca [2+] measurement was made.

Materials

O-1812 and O-2545 were obtained from Dr. Billy Martin, Virginia Commonwealth University, Richmond, VA. O-1812 was directly dissolved in Tocrisolve™, a proprietary 1:4 soya oil/water emulsion (vehicle, Tocris Bioscience, Bristol, United Kingdom). O-2545 was dissolved in phosphate buffered saline. Sutures were obtained from Sharpoint (Reading, PA). Timolol was purchased from Falcon (Napa, CA). WIN55212-2 was directly dissolved in Tocrisolve™, as was the CB1 antagonist, SR141716, and the CB2 antagonist, SR144528. SR141716 and SR144528 were generously supplied by the National Institutes of Health (Bethesda, MD). Travatan® (travoprost, 0.004%, Alcon, Fort Worth, TX), Trusopt® (dorzolamide, 2%, Merck & Co Inc., West Point, PA), and Alphagan® (brimonidine, 0.1%, Allergan, Irvine, CA) were donated by the Lion's Eye Clinic, Norfolk, VA.

Statistical Analysis

Student's t-test was used to compare data from RGC cell counts and changes in the HR, BP and ERG amplitude. A one-way ANOVA analysis was performed to compare IOP between treatments with O-1812, O-2545, WIN55212-2, timolol. Other statistical tests are specified in the results section. A difference of $p < 0.05$ was considered statistically significant. All values reported as mean ± SD unless otherwise noted.

CHAPTER V

DEVELOPMENT OF A RAT OCULAR HYPERTENSIVE MODEL

Introduction

As previously noted, it is widely accepted that an increase in IOP is a major risk factor for primary open angle glaucoma [106]. Despite some recent evidence to the contrary, the therapeutic interventions proven to consistently retard the progression of glaucoma are those that decrease IOP [107-110]. To study newer pharmacologic interventions, a valid experimental model should mimic both pathologic and physiologic characteristics of glaucoma, including sustained elevated IOP with evidence of retinal damage.

Most published animal glaucoma models elevate IOP only transiently and are therefore not suitable for long-term pharmacokinetic and pharmacodynamic studies (Table 2). Transient rapid IOP elevation is less characteristic of primary glaucoma, where gradual changes in IOP, retinal function and optic nerve morphology are pathognomonic. Currently available approaches to gradually increase IOP include occlusion of aqueous humor outflow by laser treatment of the trabecular meshwork, repeated intracameral injection of hyaluronic acid, injection of hypertonic saline or cauterization of the vortex veins (Table 2). With these approaches, a primary limiting factor is a short-lived IOP elevation that does not mimic the common clinical course, rendering these models unsuitable for long term studies.

The purpose of the present study was to devise a surgical technique that provided a constant, long term IOP elevation with concomitant evidence of retinal and optic nerve damage. We hypothesized that by minimizing surgical trauma during exposure and ligation of the vortex veins, a stable ocular hypertensive rat model suitable for long term pharmacodynamic and pharmacokinetic studies can be obtained. Further, we hypothesized that differences in strains might contribute to differences in the magnitude and duration of IOP elevation. Four strains of rats, Sprague Dawley, Brown Norway, Long Evans and Wistar, were compared to determine if some strains were more suitable models than others.

Table 2: Review of techniques to elevate IOP

Method	Strain	IOP Measuring device	Baseline IOP mmHg	Δ IOP mmHg	Duration (d, wk, or mo)	Author
Hyaluronic acid	Wistar	Tono-Pen	11.9±.9	10	5 d-10 wk	Benozzi et al.[111]
Hypertonic saline	Brown Norway	Tono-Pen	10-70	7-28	7-34 d	Morrison et al.[112]
	Brown Norway	Tono-Pen	19.5±2.8	15	39 d	Johnson et al.[113]
	Brown Norway	Tono-Pen	20.5±.5	~32	34 d	Jia et al.[114, 115]
	Brown Norway	Tono-Pen	NR*	13±7	2 mo	Chauhan et al.[116]
	Brown Norway	Tono-Pen	29	10	5 wk	Fortune et al.[89]
Laser	Wistar	Tono-Pen	19.4 ± 2.1	11-22	9 wk	Levkovitch-Verbin et al.[117]
	Wistar	Tono-Pen	15.8±.6	14	2 mo	WoldeMussie et al.[118]
	Wistar	Tono-Pen	16	16	3 wk	Hare et al.[119]
Laser + India ink	Wistar	pneumo	11±05	5.9	8 d	Karim et al.[120]
	Wistar	pneumo	14-15	10	4 wk	Ueda et al.[121]
Cautery-3 veins	Wistar	pneumo	11.5±.9	8	3 mo	Sawada et al.[122]
	Brown Norway	Tono-Pen	25.1±0.5	9	8 wk	Grozdanic et. al.[123, 124]
	Wistar	Tono-Pen	12-22	>36	3 mo	Mittag et al.[125]
Ligation	Sprague Dawley	Goldmann	15.2±1.6	5.4±1	4 wk**	Hosseini et al.[90]
	Wistar	Tono-Pen	21.0±1.8	9	7 mo	Yu et al.[126]

* NR = not reported

** Duration of the study was 4 wks but IOP was maintained for over 40 weeks in these animals

Experimental Design

Animal Model

Male rats weighing ~ 200 gm (14 Sprague Dawley, 4 Brown Norway, 5 Long Evans and 5 Wistar) underwent vortex vein ligation (Harlan, Indianapolis, IN). Surgeries were performed on the right eye (OD) while the contralateral left eye (OS) served as a normal non-operated control.

Surgical Procedure

Rats were anesthetized with acepromazine (12 mg/kg, IP, Boehringer Ingelheim Vetmedica, Inc., St. Joseph, MO) and ketamine (80 mg/kg, IP, Phoenix Scientific, Inc., St. Joseph, MO) followed by topical proparacaine (0.5%, Wilson Ophthalmic Corp, Mustang, OK) and a lateral canthotomy was performed. After visually identifying the vortex veins through the conjunctiva and overlying rectus muscles, Westcott scissors were used to incise only conjunctiva immediately surrounding the vein. Minimal blunt dissection was performed to isolate the vessel from Tenon's capsule. Once exposed, the vessel was ligated with 9-0 nylon suture. Distention of the vessel indicated a complete ligation. The same procedure was repeated on two other vortex veins. The canthotomy was closed using 7-0 silk suture with prophylactic topical administration of tobramycin (0.3 %, Bausch & Lomb Pharmaceuticals, Inc., Tampa, FL) and loteprednol (0.5%, Bausch & Lomb Pharmaceuticals, Inc., Tampa, FL). Buprenorphine (0.05 mg/kg, IP,

Benckiser Pharmaceuticals, Inc., Richmond, VA) was administered immediately after surgery and 24 hr afterwards. Sham surgeries were performed in Sprague Dawley rats (n=4) where, after lateral canthotomy, the veins were exposed but not ligated. Surgeries on New Zealand White Rabbits were performed in a similar fashion.

IOP was measured using Goldmann applanation tonometry was employed using a custom machined applanator (Haag-Streit, Bern, Switzerland). Measurements were made at the same time of day and by the same observers. IOP measurements reported are averages of two consecutive readings. Baseline IOP was measured on three separate occasions during the week before surgery. Based on the rate of increase and sustained elevation of IOP in Long Evans and Sprague Dawley rats, IOP was measured weekly in other strains for 4 weeks and monthly thereafter. Rats were lightly sedated with ketamine (20 mg/kg) and acepromazine (3 mg/kg). Although Cohen and Bohr reported Goldmann applanation in conscious Brown Norway rats, other strains were not as docile [102]. In their protocol, firm restraint was required which decreased the reproducibility of the measurements. In our series, however, even mild sedation was not without risk as mortality related to repeated sedation occurred in four of 14 Sprague Dawley rats over a 40 week period; one of four Brown Norway rats over 16 weeks, and one of five Long Evans rats over a 6 week period.

Primary Outcome Measure

Rats with a consistent difference of >5 mm Hg between the operated eye and the contralateral control eye were included in the study.

Treatment with Timolol

In the rats with sustained ocular hypertension, the response to standard antiglaucoma therapy was measured. Rats with ocular hypertension, sustained for at least 6 weeks, were treated with a single topical administration of 20 μl of timolol (0.5%, ISTA Pharmaceuticals Inc., Irvine, CA) to the hypertensive eye. IOP in the treated eye and in the untreated normal contralateral eye was measured every 30 minutes over a 2 hr period.

Scotopic ERG changes were measured only at terminal points (32 - 72 weeks) in Sprague Dawley rats with sustained IOP elevation (n=10) to determine the extent of retinal damage. Rats were dark adapted for at least 4 hr. Eyes were dilated with atropine (1%, Falcon Pharmaceuticals, Fort Worth, TX). Rats were anesthetized with acepromazine (12 mg/kg, IP) and ketamine (80 mg/kg, IP) followed by topical proparacaine (0.5%) and methylcellulose gel (GPS 2.5%, Wilson Ophthalmic Corp, Mustang, OK). Custom made AgCl electrodes (Warner Instruments, Hamden, CT) were placed on the apex of the cornea. Stimuli consisted of 10-μsec flashes of unattenuated white light (30 cd/mm) generated by a Ganzfeld bowl photo stimulator (Grass

Instruments, PS22, Quincy, MA). ERG's were recorded separately in each eye, while the contralateral eye was covered with an eye patch. ERG responses were differentially amplified (1-1000 Hz) with a driver amplifier (Grass Instruments, Model 7DAF Polygraph, Quincy, MA). Data were acquired digitally via DASYLab (Bedford, NH). The contralateral normal eye served as an age matched negative control, e.g. response in a normal, undamaged eye. Differences in amplitude of the a- and b-waves between the operated eye and the contralateral eye were calculated and analyzed. As a positive control, other rats (n = 8) were injected intravitreally with N-methyl-D-aspartate (2 μl of 10mM NMDA), which reduces the amplitude of both a- and b-waves, and ERG's were obtained 2 weeks later as described above [89].

Histology was performed on Operated and contralateral control eyes, which were enucleated and fixed in 10% phosphate buffered formalin (Fisher Scientific, Fair Lawn, NJ) for at least 1 week.

Glaucomatous changes in the fundus were observed by slit-lamp examination on anesthetized rats. Retinal images were recorded with a 3.2 mega pixel digital camera (PowerShot S1 IS, Cannon, Tokyo, Japan) retrofitted to a slit lamp (Zeiss, Germany). Full resolution images were obtained with a constant optical zoom (10 X) and processed with Metamorph™ image analysis software. Cup to disc ratios were obtained for area, height, and width using Metamorph™ image analysis. For area measurements, the outer edge of the cup and disc were selected, pixels were counted, and the cup to disc ratio was determined. For individual height and width cup to disc measurements, diameters for

each was obtained through a central plane. The ratios were then expressed as the cup diameter to the outer disc diameter.

A standard tail cuff apparatus was used to measure heart rate (HR) and blood pressure (BP) on sedated rats. A pulse amplifier (Model 29, IITC, Woodland Hills, CA) was placed in line for digital data conversion. Data were processed with a DasyLab generated analysis module.

Student's t-test was used to compare data from RGC cell counts, treatment with timolol, and changes in the HR, BP and ERG amplitude. A one-way ANOVA analysis was performed to compare IOP, heart rate and blood pressure over time between strains. Other statistical tests are specified in the results section. A difference of $p < 0.05$ was considered statistically significant. All values reported as mean ± SD unless otherwise noted.

Results

Development of Ocular Hypertension

Ocular hypertension was achieved by ligating two superior (temporal and nasal) and one inferior temporal vortex veins. Ligation of all four vessels resulted in pan vascular rupture in the anterior chamber and was not desirable. No differences were noted when the position of the ligatures was changed, for e.g. two inferior (temporal and nasal) and one superior (temporal or nasal) (data not shown). However, the superior

nasal vessel was preferred over the inferior nasal due to the presence of the nictitating membrane in this aspect and also because of its ease of access. The primary outcome measure for ocular hypertension was a consistent difference of at least 5 mm Hg between the operated eye and the normal contralateral eye. In all strains of rats, this benchmark was achieved between 1 and 4 weeks following surgery. In the week following surgery, post-surgical inflammation was occasionally noted, which resolved after treatment with topical antibiotics and steroids. No signs or symptoms of inflammation persisted beyond one week. Animals were removed from the study only due to failure to meet hypertensive criteria for more than two consecutive weeks or to adverse effects of sedation.

Sprague Dawley Rats

Sprague Dawley rats developed ocular hypertension within one week of surgery (Figure 5). Although the contralateral control was elevated at one week, it returned to baseline measurement by two weeks and remained constant throughout the study period. At two weeks, IOP in the operated eye was significantly elevated to 17.0 ± 2.1 mmHg from a baseline of 9.6 ± 0.6 mmHg. In the initial group (n=5), all rats developed ocular hypertension and remained elevated past 6 weeks. This surgery was repeated in another group (n=9) with a total of 14 rats, out of which only one was removed from the study due to failure to meet the hypertensive criteria for two consecutive weeks. Ocular hypertension in Sprague Dawley rats remained for 40 wks. In sham-operated animals (n=4), IOP remained at baseline value of 11.0 ± 1.2 mmHg to 11.3 ± 1.0 mmHg four weeks later with no IOP changes in the contralateral eye.

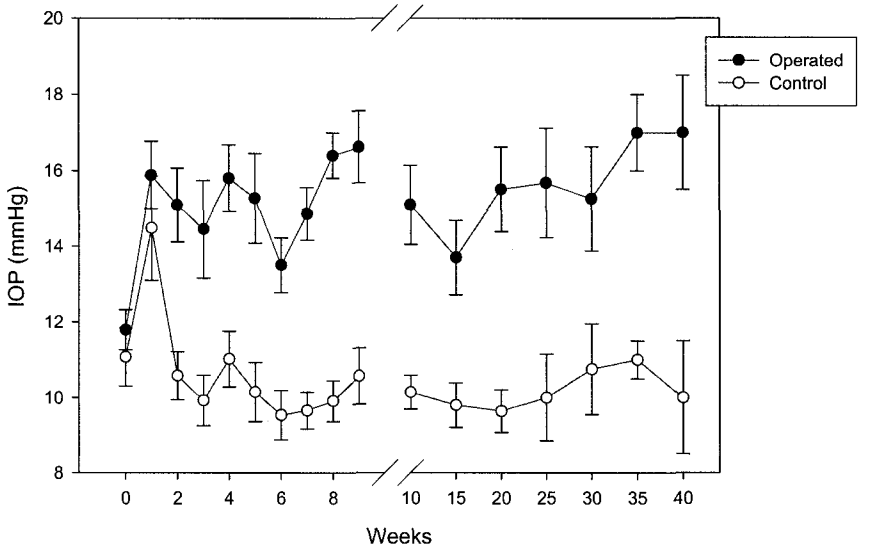

Figure 5: Vortex vein ligation in Sprague Dawley rats.
Week 0 represents baseline presurgical IOP. By 2 weeks after surgery IOP, measured with Goldmann tonometer, was significantly elevated ($p<0.05$, n=13). IOP remained significantly elevated over 40 weeks ($p<0.05$) maintaining a >5 mm Hg increase over the contralateral control eye. All controls used in this experiment were unoperated contralateral eyes. All pair wise comparisons conducted using the Student's t-test.

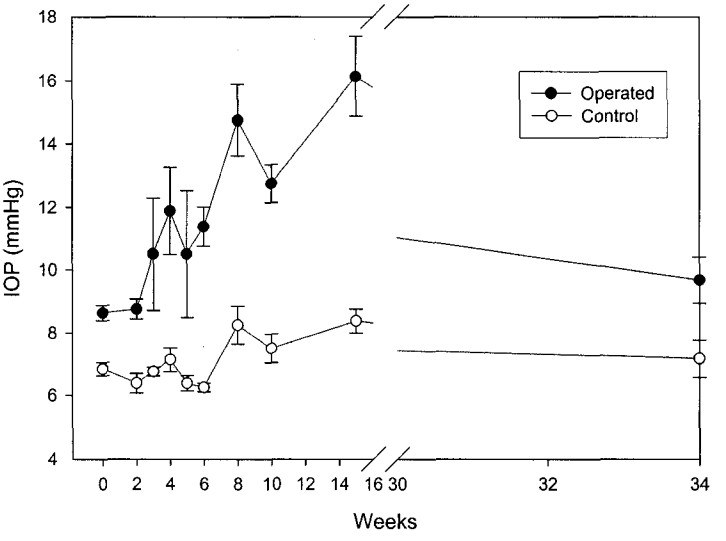

Figure 6: Vortex Vein ligation in Brown Norway rats.
IOP was significantly elevated in operated eyes by 3 weeks following surgery ($p<0.05$, n=4). However after 15 weeks IOP gradually decreased and no significant IOP elevation was seen after 34 weeks in the operated eye compared to contralateral control eye. All pair wise comparisons conducted using Student's t-test.

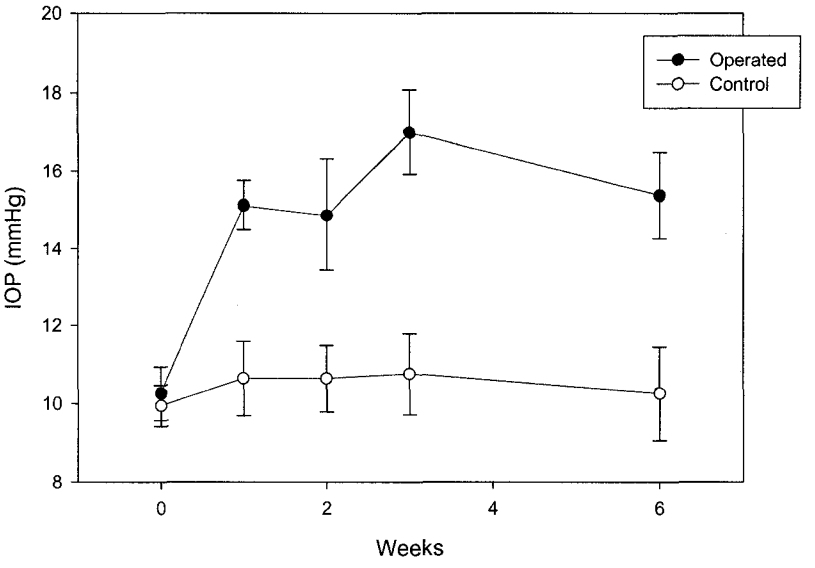

Figure 7: Vortex vein ligation in Long Evans rats.
Long Evans rats showed a rapid response to episcleral vein ligation as IOP was significantly elevated by 1 week ($p<0.05$, n=5). Operated eyes maintained a > 5 mm Hg increase over contralateral control eyes for 6 weeks ($p<0.05$). However, measurements were suspended after 6 weeks due to persistent, rapidly progressing conjunctival vascularization. All pair wise comparisons conducted using Student's t-test.

Brown Norway Rats

Three weeks following surgery, IOP was significantly elevated in Brown Norway rats to 10.5±1.8 mmHg from a baseline of 6.8±0.1 mmHg (Figure 6, n=4). Peak IOP at 15 weeks was 16.1±1.3 mmHg but was not maintained afterwards. Fifteen weeks after surgery, one rat failed to meet the inclusive criteria and by 35 weeks none met the hypertensive criteria. No significant anatomical differences were seen in Brown Norway rats as veins were easily located lateral to the rectus muscles and were similar in size and shape to the Sprague Dawley rats.

Long Evans Rats

In Long Evans rats, ocular hypertension developed within one week (Figure 7, n = 5). By 3 weeks one rat failed to meet the criteria for ocular hypertension. While all Long Evans rats developed ocular hypertension, IOP remained elevated for only 6 weeks. Of particular note was the development of rapidly progressing conjunctival vascularization, even when subjected to minimal surgical manipulation. Within 6 weeks, this vascularization had extended to the limbus resulting in the termination of the study.

Wistar Rats

Vortex vein ligation in Wistar rats was met with several surgical complications. Significant anatomical differences, mainly, their smaller sized globes and difficulty in locating the vortex veins contributed to these failures. Due to these reasons the surgical procedure was slightly modified to include cauterization of the veins. Even after this modification, the change in IOP did not meet the criteria for ocular hypertension and was inconsistent and short-lived. After one week IOP increased from 10.5±1.7 mmHg to only 11.5±0.7 mmHg. By week 4, IOP was 13.1±3.2 mmHg in two rats. However, all rats gradually succumbed to sedation related respiratory complications and died within 6 weeks. During autopsy, numerous collateral arteries bypassing the ligation were noted.

Heart rate and blood pressure

Heart rate and blood pressure were measured in each strain of rats before surgery (e.g. baseline) and in the weeks following surgery. Four weeks after surgery, heart rate and blood pressure were unchanged from the presurgical baseline with one exception (Table 3). Systolic blood pressure was significantly increased in the Long Evans rats and diastolic pressure tended to decrease. There were differences between the strains, but they were strain specific since these differences were reflected in baseline data. For example, presurgical heart rate in Long Evans rats was significantly higher than in the other three strains.

Table 3: Heart rate and blood pressure measurements in rats

Rat Strains		Heart Rate bpm	Systolic BP mmHg	Diastolic BP mmHg	N
Sprague Dawley	Postop*	423.4 ± 39.7	120.2 ± 15.0	94.6 ± 14.7	7
	Preop	*383.1 ± 97.1*	*134.2 ± 38.3*	*94.4 ± 44.6*	*4*
Brown Norway	Postop	412.8 ± 20.8	119.7 ± 16.5	94.4 ± 12.1	4
	Preop	*409.5 ± 22.9*	*118.4 ± 15.0*	*94.6 ± 8.3*	*3*
Long Evans	Postop	498.3 ± 17.3 †	156.8 ± 6.6 ‡	104.3 ± 6.9	4
	Preop	*508.6 ± 34.2*	*137.1 ± 5.3*	*115.7 ± 10.2*	*4*

Heart rate and blood pressure remained constant for each strain for the duration of the experiment; preop values were not obtained on all rats.
* Postop = 4 weeks after surgery in the presence of elevated IOP
† significantly different from other strains $p < 0.05$.
‡ significantly different from baseline, $p < 0.05$.

Electroretinogram (ERG)

Sprague Dawley rats with sustained ocular hypertension were examined by ERG and fundus photography to ascertain whether the sustained increase in IOP resulted in glaucomatous changes to the retina. ERG's were performed on both eyes at terminal points (32 - 72 weeks). The amplitudes of the a-wave and b-wave in the hypertensive eye were compared with the contralateral normotensive eye. While no ERG aberrations were seen up to 12 weeks, after 30 weeks the a-wave amplitude significantly decreased by 45.9±22 % ($p = 0.03$) and the b-wave by 34.9±24% ($p < 0.03$) from that measured in the normotensive contralateral eye (n=13). Statistical analysis revealed close correlations between the amplitudes of the a- and b-waves with both IOP and retinal ganglionic cell loss (Table 4).

In normal rats, injection of NMDA diminished the amplitude of both a- and b-waves in a manner similar to the surgically ligated eyes. Two weeks after injection, the a-wave amplitude significantly decreased to 55±0.6% ($p < 0.002$) of the preinjection amplitude. Concomitantly, the b-wave amplitude decreased to 49±0.8% (n=8, $p = 0.02$). The ERG wave amplitude in the contralateral control eye did not deviate significantly throughout the observation period ($p<0.521$).

Table 4: Correlation analysis of ERG amplitudes (a- and b-wave), increased IOP and RGC loss.

	a-wave		*b-wave*	
	r^2	*p*	r^2	*p*
Increased IOP	*0.992*	*<0.001*	*0.992*	*0.008*
RGC loss	*0.945*	*0.05*	*0.829*	*0.008*

Histology

Representative retinas from Sprague Dawley rats with sustained ocular hypertension were analyzed for morphological changes. The retinal cell counts were obtained from retinal cross-sections at the optic nerve level. Three of 14 ligated eyes with sustained IOP elevation were sectioned. While the overall retinal thickness remained constant in the ligated and control eyes, reduction in density of the inner and outer plexiform layers, and the retinal ganglionic layers was noted in the ligated eyes. Retinal ganglionic layer cell density was significantly reduced to 46.5 ± 3.9 (cells per 200μm) compared to 62.5 ± 4.5 in the contralateral control, a 26% reduction ($p<0.05$). A significant correlation between increased IOP and RGC loss was noted ($r^2 = 0.946$, $p = 0.05$). Further, the ganglionic cell layer (GCL) appeared to be disrupted in the ligated eyes (Figure 8a) compared with the contralateral (control) eye, where all the retinal layers were intact (Figure 8b). In retinal cross-sections, significant cupping was observed in 3 of 4 ligated eyes (Figure 9a) compared to the contralateral control (Figure 9b). Also, enlargement of choroidal vessels was noted in all ligated eyes analyzed (Figure 10a) compared to the contralateral control (Figure 10b). No iris synechia or profound angle closure was seen in hypertensive eyes.

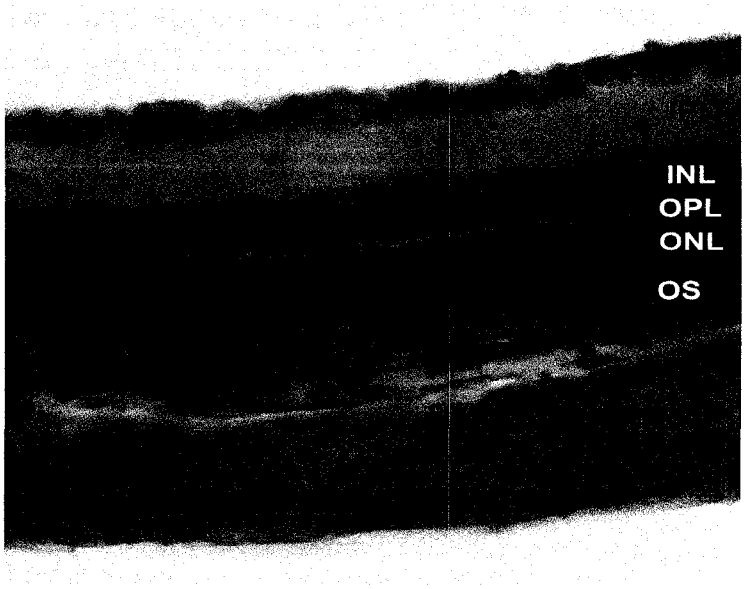

Figure 8A

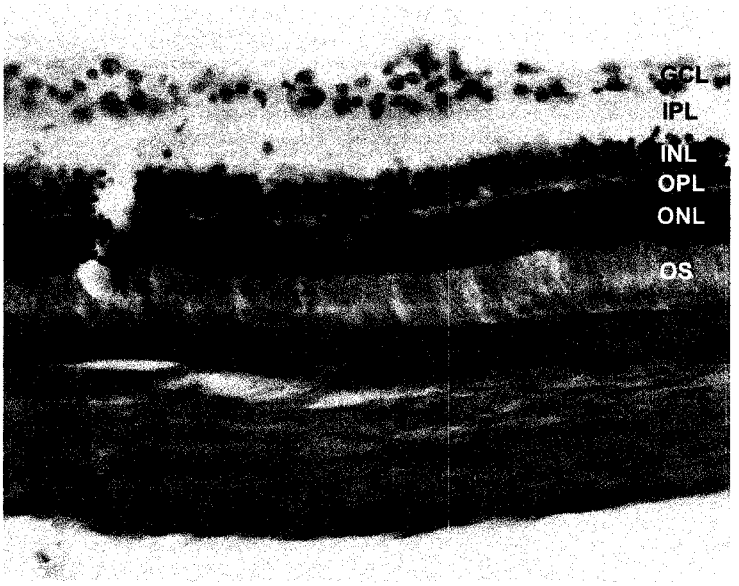

Figure 8B:

Figure 8: Representative retinal cross sections of ligated and contralateral control eyes at the level of the optic nerve.
All sections were digitally photographed at 20X magnification. *GCL* - ganglionic cell layer, *IPL* – inner plexiform layer, *INL* – inner nuclear layer, *OPL* – outer plexiform layer, *ONL* – outer nuclear layer, *OS* – outer segments. **A.** Operated eye. **B.** Contralateral control eye.

Figure 9A:

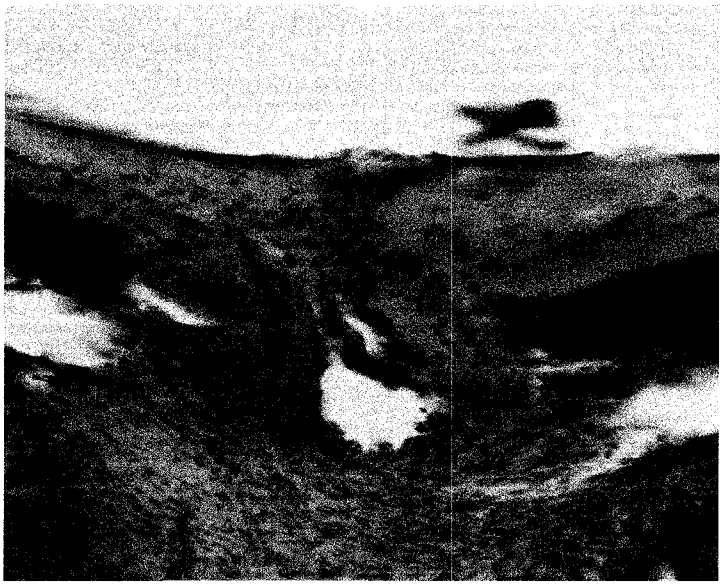

Figure 9B:

Figure 9: Retinal cross-sections representing cupping of the optic disk.
A. Cupping in the ligated eye. **B.** No cupping in the contralateral control eye. Histological analysis was performed 32-72 weeks after ligation surgery. All sections were digitally photographed at 20X magnification.

Figure 10A:

Figure 10B:

Figure 10: Anterior segment analysis of Sprague Dawley rats that have maintained IOP elevation past 32 weeks.
Although no iris synechia or profound angle closure was observed, significant choroidal vessel dilation was present in ligated eyes (**A**) indicated by arrow, compared to normal vasculature in the contralateral control (**B**) indicated by arrow.

Fundus Examination

Pathologic changes in retinal structure were evident in the Sprague Dawley group. Within 30 weeks after surgery, there was a greater than 35% increase in optic cup area of the hypertensive eye compared with contralateral normal eye. There was also a 14% increase in cup to disc ratios as measured by both width and height. The cup to disc ratios for both width and height increased to 0.65±0.045 and 0.66±0.045, respectively in the operated eye when compared to 0.57±0.07 width and 0.58 ±0.06 height in the control eye *($p<0.05$)*. The optic disc of the operated eye remained circular determined by a width to height disc diameter ratio of 1.01. A decrease in retinal vasculature was also apparent. A significant correlation was noted between IOP and cup to disk ratios for width ($r^2 = 0.908, p < 0.05$) and height ($r^2 = 0.901, p < 0.05$). By comparison, fundus examination of the contralateral eye was normal. The optic disc was circular as determined by the width to height disc diameter ratio of 1.01. There was no substantial cupping.

Corresponding changes were not observed in the Long Evans or Wistar rats where the duration of increased IOP was less than six weeks. Due to their pigmentation, the optic disc margin was not easily demarcated in Brown Norway rats. However, 14 weeks after surgery, the morphology of optic disc and retinal vessels had not changed from their presurgical appearance.

Response to timolol

To ascertain if the operated eyes respond to standard antihypertensive therapy, a single dose (20 μl) of timolol 0.5% was administered to the hypertensive eye. IOP was measured every 30 minutes over a 2 hr period (Figure 11). Within 0.5 hr, in Sprague Dawley rats timolol significantly decreased IOP from baseline and it remained significantly decreased for over 2 hr ($p<0.05$). Timolol decreased the IOP to the extent that it was no longer different than that of the contralateral eye ($p= 0.77$). In Brown Norway rats IOP initially decreased from pretreatment values ($p<0.05$), but the IOP remained greater than that in the contralateral eye ($p < 0.01$). Long Evans rats had the least response to timolol treatment. At 0.5 hr, there was only a 2 mmHg difference between eyes, and IOP remained significantly higher than in the contralateral eye ($p < 0.01$). No drug crossover IOP effects were observed in contralateral eyes of any strain throughout the study period.

Discussion

As new pharmacologic approaches for treatment of glaucoma emerge, demonstration of efficacy and comparison with current therapy requires suitable preclinical models. Although the specific etiology of primary glaucoma is not well understood, increased resistance to aqueous humor outflow from the eye causes IOP to increase. As ocular hypertension remains a primary risk factor, most models use an increase in IOP as their primary end point.

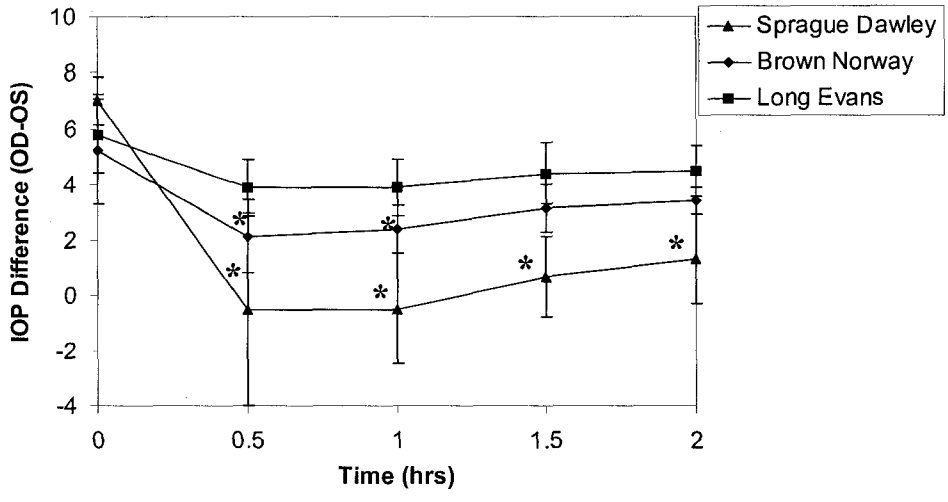

Figure 11: Effects of timolol (0.5%, 20µl) on IOP were measured over a 2 hr period in three rat strains with surgically elevated IOP.
Sprague Dawley rats responded rapidly with a significant decrease at 0.5 hr and lasting 2 hr ($p<0.05$). The response of Brown Norway rats was moderate with a significant decrease only at 0.5 hr and 1 hr ($p<0.05$). Long Evans rats had small but not significant decrease in IOP. All data are mean ± SD (n=4/group), all comparisons conducted by ANOVA analysis. Data at 0 hr represent baseline values before administration of timolol.
* Significant compared to baseline, $p<0.05$

Both surgical and nonsurgical approaches have focused on increasing resistance to aqueous humor outflow. When hyaluronic acid was injected intracamerally in Wistar rats, IOP increased, but only for 8 days; multiple injections were required to sustain the increase [111]. Karim et al elevated IOP by argon irradiation of rat trabecular meshwork in Wistar rats [120]. Also, Levkovitch-Verbin et al used a diode laser aimed at the trabecular meshwork and vortex veins which raised IOP in Wistar rats for 9 weeks [117].

With only moderate success of the nonsurgical approaches to sustain ocular hypertension, surgical intervention has been more successful. After cauterization of 3 vortex veins and 2 episcleral veins in Brown Norway rats, only 36% developed elevated IOP with duration of 5 weeks [124]. Cauterization of 3 episcleral veins increased IOP 1.6 fold over control in Wistar rats, but required re-cauterization after 6 months [122]. In our study, compared to cauterization, minimally invasive vortex vein ligation resulted in significant IOP elevation that lasted at least a year. In addition, the success rate for developing elevated IOP was higher (~90% for Sprague Dawley rats). Therefore, ligature of the vortex veins yields in a consistent and longer lasting ocular hypertension.

The development of our surgical technique to achieve a sustained elevation of IOP required several iterations. Unfortunately, complete dissection to expose the vortex vessels caused anterior segment ischemia manifested as erythema, corneal opacification and central ulceration that did not improve with tarsorrhaphy and antibiotics. Ocular hypotension frequently occurred, probably due to disruption of perfusion to the iris major arterial circle, as well as aqueous humor production by the ciliary body. This

response is analogous with the anterior segment ischemia that occurs in patients after strabismus surgery when multiple rectus muscles are disinserted.

These unsatisfactory surgical results prompted us to develop a technique, in which careful attention was given to minimizing surgical trauma, primarily by identifying the vortex vessels directly through the conjunctiva and then ligating them using only very small minimal incisions. We hypothesized that the greater the surgical insult, the faster and more extensive the development of collateral circulation. It was crucial that incisions of the conjunctiva be limited to the immediate area of the vortex veins. Ligation rather than cautery of vortex veins is essential. Collateral circulation was more extensive when cautery alone or cautery following ligation was used. It is possible that the use of cautery alone is a sufficient local insult to stimulate the formation of collaterals. Similarly, in other studies where only cautery was used, the duration of ocular hypertension was shorter than that following vortex vein ligation [122].

Following ligation of three of the four vortex veins, a steady elevation was achieved without negative impact on other ocular structures except for the retina. All possible ligation combinations were attempted, but superior (nasal and temporal) and inferior temporal vessels were most commonly ligated due to their ease of access. The inferior nasal vein was most difficult to access and caused excessive damage to surrounding tissues during ligation. Ligation of all four vortex veins resulted in infarcted blood vessels due to almost complete occlusion of the outflow facility. Excessive occlusion of the outflow apparatus and stimulation of angiogenic factors promotes

formation of anastomoses or collateral circulation [127, 128]. This stimulus is amplified by surgical trauma; in our study the surgical trauma was limited to the immediate area of the vortex veins to preserve much of the normal anatomy of the eye. These novel modifications in the surgical procedure improved the performance of the ocular hypertension model. Using a similar technique, Yu et al reported an IOP elevation of approximately 9 mmHg in Wistar rats over the control eye that was compromised by collateral formation in 60% of the cases [126]. Subsequently, repeated ligations were necessary to maintain the IOP elevation reported to be maintained over a 7 month period.

Although the increase in IOP was of similar magnitude in Long Evans and Brown Norway rats, these strains are less suitable models than the Sprague Dawley rats. In both Long Evans and Brown Norway rats, the elevated IOP was not sustained and optic nerve damage minimal. Rapid formation of anastomoses and postoperative complications further compromised the Long Evans rat model. The Long Evans rats did not respond to timolol and the response in the Brown Norway rats was small and of short duration. In both strains morbidity associated with sedation was high. If increased IOP could be sustained, Brown Norway rats have potential as a model due to the ease of observing changes in retinal and optic nerve morphology. Preliminary studies with Wistar rats were similarly unproductive. Wide variations in vortex vein anatomy and sedation-related morbidity precluded inclusion of a suitable number of animals.

It was not surprising that the responses varied between the rat strains because there are considerable differences in the biochemical, pharmacological and neuronal

responses among rat strains [129, 130]. Although rats in these experiments were sex and age matched, certain differences, such as response to anesthesia and surgery, persisted. Strain dependent neovascularization and development of collateral circulation could be attributed to innate regulators of vascularization. Compared to Sprague Dawley rats, retinal ischemia in Brown Norway rats produces substantially more VEGF [131]. Nitric oxide synthetase and glutamate activity is strain dependent, which may have important implications for the extent of glaucomatous retinal damage developed in different strains [132]. Brown Norway rats also have impaired autoregulation of blood pressure compared to Long Evans or Sprague Dawley rats, which may explain their poor response to beta-adrenergic blockade [133]. If blood pressure in Brown Norway rats does not respond normally, regulation of IOP may also be atypical, since diurnal variation in IOP correlates directly with diurnal variations in blood pressure [134].

Additionally, this technique was also performed on New Zealand White rabbits (data not shown). Ligation of three vortex veins failed to elevate IOP, while ligation of all four caused excessive IOP elevation and rupture of veins. In ligated rabbits (three vein), development of significant collateral circulation was noted (Figure 12) which could contribute to lack of IOP elevation. Therefore, it can be concluded that this strain of rabbits is not suited for this technique as they have a greater potential to develop collateral circulation.

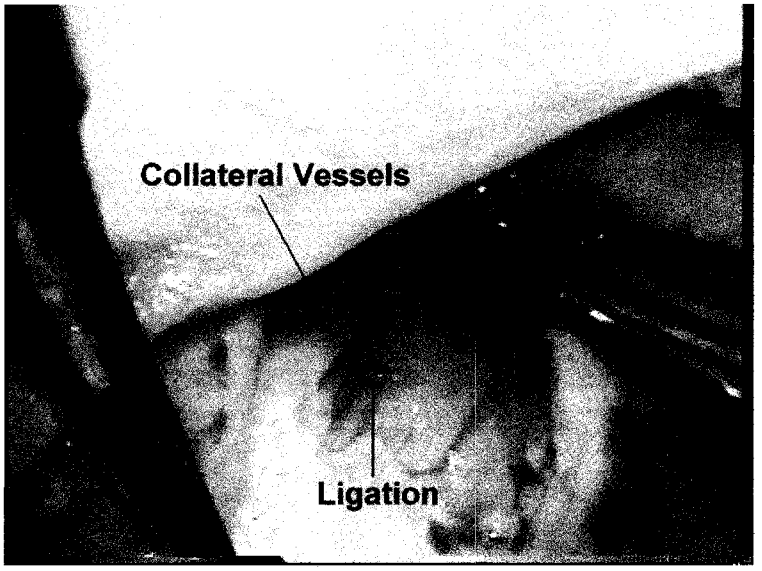

Figure 12: Vortex vein ligation surgery performed on New Zealand White Rabbits. No IOP elevation was produced in the operated eyes due to significant collateral vessel formation within 2 weeks of surgery.

Once the effectiveness in maintaining ocular hypertension was established, we examined whether this model mimicked other salient factors associated with glaucoma. In our model significant loss of both a- and b- wave amplitudes were noted 32 weeks after surgery. The loss of retinal ganglionic cells and diminished amplitude of ERG wave pattern is synonymous with glaucomatous damage [89]. While a-wave changes are pathognomic, the decrease in b-wave amplitudes raise concerns of possible ischemic involvement and choroidal hypertension in the ensuing retinal damage. With any occlusive surgical procedure, a potential for ischemic damage exists. With vortex vein ligation, dilation of choroidal vessels leads to IOP elevation that is gradual and prolonged and does not directly affect central vessels of the eye. Therefore any resulting ischemic insult is not as severe in comparison to damage induced by ligature of optic vessels [135]. B-wave deficits are also observed in DBA/2NNia mice model of angle closure glaucoma [136]. In this genetic model both a- and b-wave amplitudes decreased after 8 months, thus sharing many similarities with human glaucoma, however the specific causes of the ERG changes in DBA/2NNia mice is not clear. Following other procedures, such as injection of ICG dye in the anterior chamber with a rapid onset of ocular hypertension, it would be expected that ischemic injury would be a major contributor to retinal damage. In these models, b-wave damage is prominent in comparison to the inner retinal function [124]. In all cases, the glaucomatous condition presents with both a- and b-wave deficits and contributions by ischemia and choroidal hypertension cannot be ruled out. In addition to the significant retinal functional damage, pathologic structural changes were observed in retinal vasculature and the optic disc. Although all measured parameters (area, height

and width) of the cup and disc increased in the operated eye, the disc remained circular, indicative of early glaucomatous changes.

The results of the present study help to define optimal conditions to create an effective rat ocular hypertensive model. While IOP increased gradually in most strains, only in Sprague Dawley rats was it sustained for up to a year. Retinal degeneration was coincident with the sustained increase in IOP. The Sprague Dawley rat model was also remarkably free of postoperative complications and responded well to standard pharmacotherapy with timolol. Indeed, we have recently used this Sprague Dawley model to demonstrate that repeated topical administration of the cannabinoid agonist WIN-55-212-2 significantly decreased IOP without developing tolerance [90]. In conclusion, the results of the present study indicate that ligation of three of four vortex veins in the Sprague Dawley rat is an optimal preclinical rat model to investigate pharmacological treatments for intraocular hypertension.

CHAPTER VI

OCULAR PHARMACOKINETICS OF O-1812 AND O-2545 IN AN ARTIFICIALLY PERFUSED RAT EYE

Introduction

A major interest in ocular pharmacology has been the description and improvement of therapeutic drug absorption in the compartments of the eye. One of the primary factors affecting a drug's pharmacokinetic profile is its lipophilicity. It is well known that lipophilic compounds can more easily cross biologic membranes, and thus, can more easily reach their sites of action. Cannabinoids are inherently lipophilic, especially O-1812. Lipophilic compounds are difficult to solubilize and to dissolve frequently require solvents like ethanol and DMSO. Moreover, lipophilic compounds tend to precipitate out of solution and have a limited shelf life.

While hydrophilic compounds would be safer to administer, their absorption profiles must be comparable to that of lipophilic compounds. Measurements of intraocular drug concentrations over time are limited, however, by the amount of aqueous or vitreous samples that can be obtained.

A relatively new method for the elucidation of pharmacokinetic profiles *in vivo* is microdialysis. It has been successfully utilized for regional sampling of low-volume

compartments of many tissues, such as the brain, joints, kidney, and eye [93]. By taking a minute amount of aqueous humor and re-perfusing an equivalent volume, microdialysis sampling allows for serial measurements of the aqueous fluid in order to create a complete concentration versus time curve for an individual subject. Rittenhouse et al used microdialysis sampling to produce intra- and inter-animal absorption profiles of propranolol after topical and intracameral injection, showing it to be an accurate and easily reproducible method [93]. In this study, we will use a similar method to examine the pharmacokinetics of lipophilic and hydrophilic O-compounds after topical ocular administration.

Experimental Design

Animal Model

Male rats weighing ~ 200 gm (14 Sprague Dawley, 4 brown Norway, 59 Long Evans and 5 Wistar) underwent vortex vein ligation. All studies were conducted in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research, and were approved by the Eastern Virginia Medical School (EVMS) Institutional Animal Care and Use Committees.

Surgical Model

An adult male Sprague Dawley (SD) rat was euthanized by IP injection of Fatal Plus in accordance with IACUC (Institutional Animal Care and Use Committee) regulations. Placed in supine position, Y incision was made to expose chest cavity. Once descending aorta was exposed and isolated, the inferior aorta was sutured closed,

superiorly to the suture but inferior to the aortic arch. A window into the lumen of the aorta was made and tubing placed securely inside; held with a suture and surgical glue. At the end proximal to the heart, the ascending aorta was sutured closed. Once a closed system was achieved, profusion began and maintained IOP during cannulation without elevation of IOP. Modified 27 gauge needle accompanied by the cannulation apparatus was inserted into the OS anterior chamber. OD received the same insertion technique using the 27 gauge needle into the OD vitreous chamber. Perfusion with 1X PBS was maintained for a period with stability, topical drug was added and samples taken every 10 minutes for 2 hours for analysis by HPLC.

Cannabinoid concentration was quantified by HPLC. Using a modification of the method described by Barberi-Heyob and colleagues, dialyzed aliquots were injected on a 5-um reverse-phase column (Prodigy 5 um ODS(2) analytical column, 150 x 4.6 mm, Phenomenex, Torrance, CA) at ambient temperature with a mobile phase of acidified acetonitrile water (pH = 4.0) solution [94, 95]. The flow rate was 1 mL/min with UV detection (254 nm). Chromatograms were analyzed using Beckman System Gold software (Beckman Instruments, Inc., Fullerton, CA).

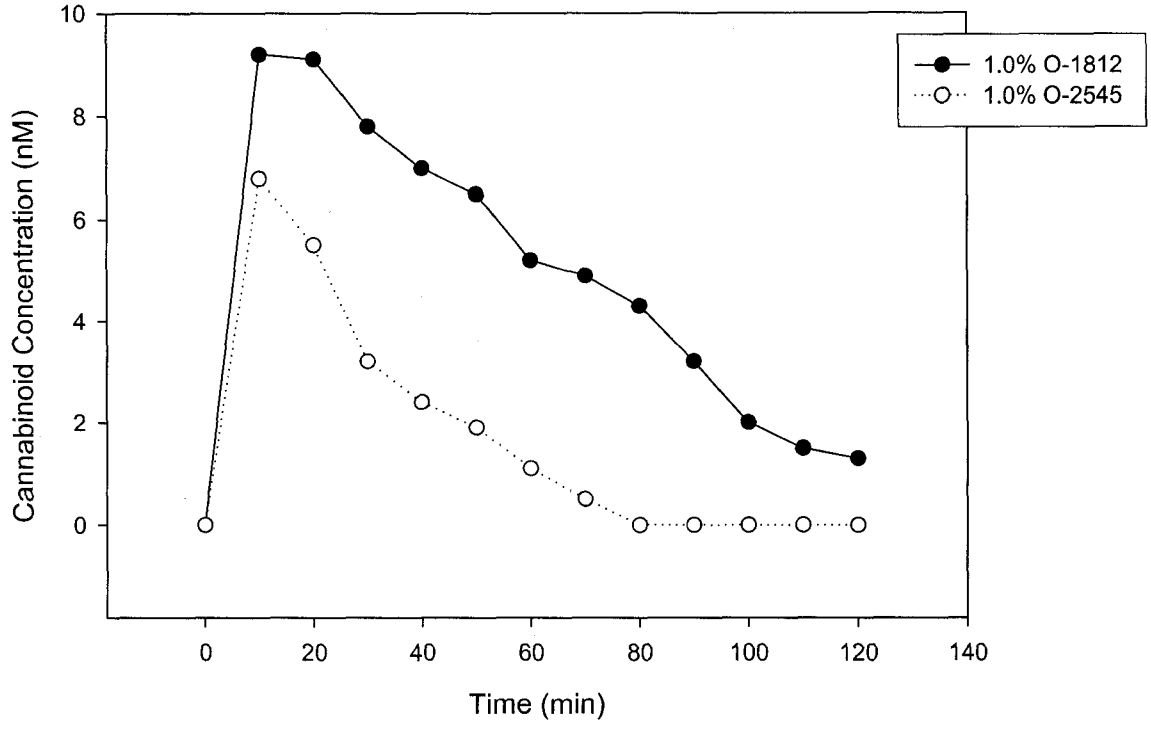

Figure 13: Pharmacokinetic profile of O-1812 and O-2545 in the anterior chamber of an artificially perfused rat eye.
Eyes were dialyzed for 120 min with a sample taken every 10 min.

Results

Lipid soluble O-1812 more effectively penetrates the anterior chamber

Cannabinoid concentration as a result of topical administration was assessed in the anterior chamber of a rat eye (Figure 13). After artificial perfusion, dialysates were collected every 10 min and analyzed with an HPLC. Both O-1812 and O-2545 rapidly crossed through the cornea into the anterior chamber of the eye. Lipid soluble O-1812 was more effective than water soluble O-2545 in penetrating the anterior chamber, a difference of 2 nM was noted (n=1 for each drug). Cannabinoid concentration decreased over time. After 80 min, there was no trace of O-2545; however O-1812 concentration was greater than 2 nM for 120 min.

Lipid soluble O-1812 more effectively penetrates the posterior chamber

Cannabinoid concentration after topical administration was assessed in the posterior chamber of a rat eye (figure 14). Cannabinoid transport to the posterior chamber was not as rapid as compared to the anterior chamber. Concentration of O-1812 in the posterior chamber was 5 nM after 30 min and decreased to 2 nM after 90 min where it remained for the duration of the study (120 min). In contrast, maximal concentration of O-2545 was 3 nM after 30 min, but was eliminated after 80 min. After a single topical dose of O-1812 or O-2545, lipid soluble O-1812 more effectively penetrated the posterior chamber than water soluble O-2545.

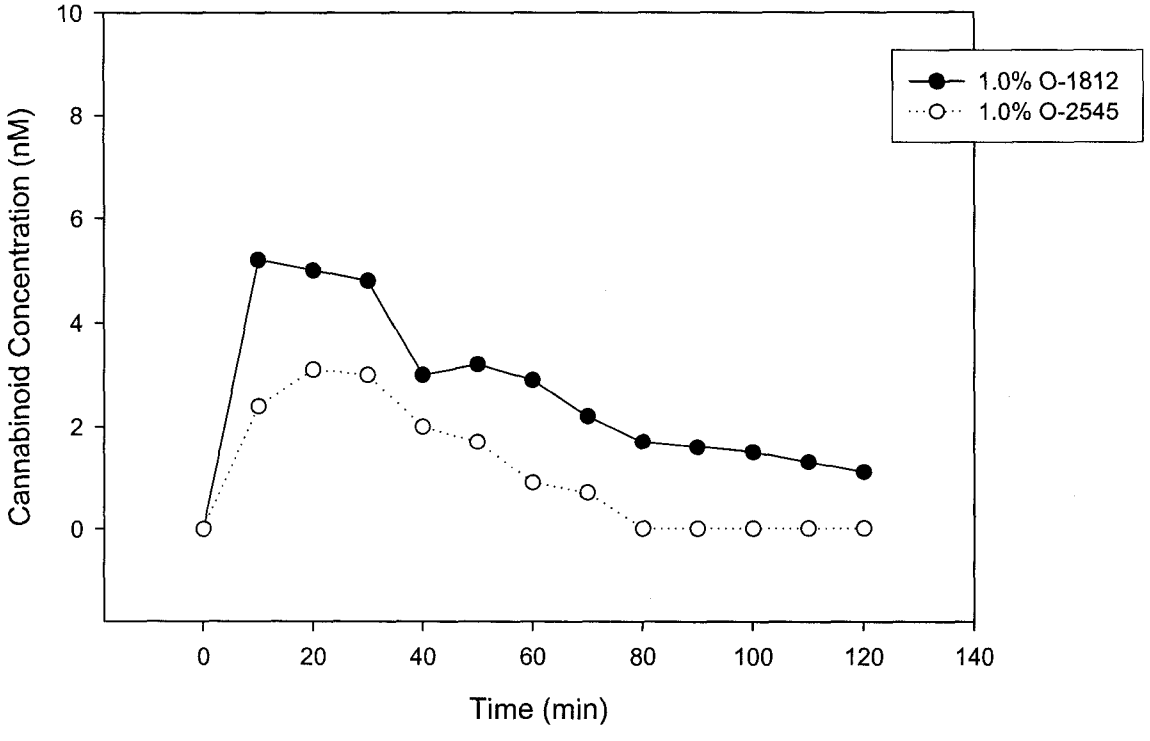

Figure 14: Pharmacokinetic profile of O-1812 and O-2545 in the posterior chamber of an artificially perfused rat eye.
Eyes were dialyzed for 120 min with a sample taken every 10 min.

Discussion

Compared to other tissues, the aqueous and vitreous chambers are relatively privileged sites. Compared with systemic administration, where drug plasma concentration is often sufficient to obtain therapeutic levels in a given region, it is difficult to do so in the eye because certain drugs do not cross the blood-vitreous barrier [137]. However, certain substances are more suited than others to cross the blood-vitreous barrier, namely, lipophilic compounds. Mayers et al compared the intraocular concentrations of lipophilic chloramphenicol versus hydrophilic amikacin after intravenous administration [138]. They demonstrated that chloramphenicol reached higher concentrations in the anterior chamber than did amikacin. Moreover, chloramphenicol had a rate of elimination from the aqueous humor identical to its rate of elimination from the serum, while amikacin had a lower peak aqueous concentration and was more quickly eliminated.

Lipophilic drugs can reach therapeutic levels in the eye when administered intravenously, although high doses are required. Mayers et al found that, when directly injected into the eye, chloramphenicol and amikacin were both eliminated more quickly, and the two compounds had comparable half-lives [138]. They attributed this to the lack of a membranous barrier to the drainage mechanism of Schlemm's canal, which abolishes the lipophilic compound's absorptive advantage. While intravitreal injection might hold promise for improved concentration curves of hydrophilic compounds, it is not a clinically desirable option due to patient discomfort, anxiety, and increased risk of infection.

The ideal mode of administration of ocular medication is topically. Drops can be applied easily by the patient in almost any setting. Studies have shown, however, that topically-administered drugs achieve much lower concentrations than those injected intraocularly [93]. To evaluate how the concentrations of these drugs affect their action, an accurate description must be made of its pharmacokinetic profile. This can be done in a number of ways. One method is to take serial samples of aqueous humor from the same subject. The volume of fluid available for sampling is limited, however. While this might be overcome by a "washout" period in between treatments to replenish aqueous fluid and clear the experimental compound, repeated cannulation of the sclera could lead to inflammation and scar tissue formation, which would affect sampling. Another technique involves taking samples of fluid from a large number of subjects at staggered time intervals after administration of the experimental compound and combining all the data into one concentration vs. time curve. This can be skewed by variability between individual subjects, however.

Several methods have been employed to accurately model concentration curves in the eye. Drusano et al attempted to create a reliable curve of plasma concentrations vs. vitreous concentrations of ciprofloxacin using single measurements of intraocular concentrations from multiple subjects and a population pharmacokinetic modeling program [137]. While this allows for fewer vitreous samplings, repeated plasma samplings are required; furthermore, it is based on data compiled from different subjects which may be skewed by inter-subject variation. Mayers et al utilized a method consisting of multiple paracenteses to observe the absorption of antibiotics [138]. By beginning to sample

from different subjects at different time intervals, they showed that the number of samples taken do not affect clearance of the drug from the aqueous humor. This method required many subjects, however, and did not rule out the possibility of an occult inflammatory process affecting the clearance of the antibiotics.

Using *in vivo* microdialysis, a pharmacokinetics of lipid and water soluble cannabinoids can be made with continuous sampling. Since these cannabinoids are newly synthesized, their pharmacokinetic profiles have not been elucidated. After a single topical administration, lipid-soluble O-1812 effectively penetrated both the anterior and posterior chamber and was maintained for at least 120 min. O-2545, being water-soluble, did penetrate both the ocular chambers, but was eliminated more rapidly than the lipid soluble O-1812.

CHAPTER VII

TOPICAL APPLICATIONS OF NOVEL CANNABINOIDS, O-1812 AND O-2545, REDUCED INTRAOCULAR PRESSURE IN A RAT OCULAR HYPERTENSIVE MODEL

Introduction

IOP is the most important risk factor for the development of glaucoma, a leading cause of blindness world wide [21, 139, 140]. Consequently, the reduction of IOP is recognized as the main treatment to reduce visual loss in glaucoma patients. Several multicenter clinical trials have demonstrated that reducing IOP retards the progressive visual field damage in patients with primary open angle glaucoma or normal tension glaucoma [1, 36].

A variety of naturally occurring and synthetic cannabinoid agonists have been evaluated for their ability to reduce IOP in humans and laboratory animals [141-143]. Most bind both cannabinoid receptors, the CB1 receptors which are predominantly localized in the CNS [63] and the CB2 receptors identified in the periphery and associated with immune function [144]. It has been suggested that the IOP reducing effects of cannabinoid agonists are related to their actions on the CB1 receptor, whose presence has been demonstrated in the ciliary body, iris, trabecular meshwork, and retina [13, 16, 44, 52]. Stimulation of these CB1 receptors is hypothesized to decrease IOP and, consequently, may retard the progression

of glaucoma. Recently, CB2 receptors in the trabecular meshwork have also been implicated in cannabinoid-mediated IOP reduction [53].

The therapeutic usefulness of systemic cannabinoids is limited by their well-described psychomimetic and systemic effects; however, topical drug delivery may reduce these problems. The primary purpose of the present study is to determine whether acute topical administration of O-1812, O-2545 is comparable to WIN 55212-2, a well studied high efficacy aminoalkylindole cannabinoid analog. With topical application, undesired systemic and ocular effects can be obviated while maintaining efficacy. The efficacy of O-1812, O-2545, and WIN 55212-2 was evaluated in rats with high IOP, following surgical occlusion of three vortex veins. As an additional measure, receptor (CB1 or CB2) dependency of the IOP effect in a rat chronic ocular hypertensive model was studied by using selective antagonists, SR 141716A or SR 144528, for each respective cannabinoid receptor.

Experimental Design

Sprague Dawley rats were randomly assigned to either O-1812, O-2545, WIN 55212-2, or Tocrisolve™ (vehicle) treatment groups. Only O-1812 and WIN 55212-2 were dissolved in Tocrisolve™ while O-2545 was water soluble and dissolved in PBS. These compounds were applied topically to the hypertensive right eye. The vehicle served as the negative control while WIN 55212-2 was a positive control. All animals were allowed a minimum washout period of 1 wk between subsequent experiments. The left eye (OS) served as the untreated control. At the beginning of each experiment,

baseline IOP, heart rate (HR), and blood pressure (BP) measurements were obtained. IOP measurements were repeated at 30, 60 and 120 min after drug administration, in the treated eye (OD), as well as in the contralateral untreated eye (OS). HR and BP were also measured at 30, 60 and 120 min after drug administration. Before and at the conclusion of each experiment, all eyes were examined by slit lamp for signs of ocular irritation and by in vivo confocal microscopy for changes in corneal thickness.

Experiments using CB1 antagonist, SR141716, or CB2 antagonist, SR 144528, were performed to determine receptor specificity of these compounds. The CB antagonists were prepared by dissolving 50 μg of compound in 50 μl of Tocrisolve™ (0.1%). To get maximal effect, the antagonists were applied topically 30 min prior to the administration of the cannabinoids. The CB1 antagonist was SR141716 (Ki [CB1] = 5.6 nM, Ki [CB2] >1000 nM) [145] and the CB2 antagonist was SR 144528 (Ki [CB2] = 0.6 nM, Ki [CB1] = 437 nM) [146].

Additional experiments to test for the confounding effects of sedation on IOP were performed. In this experiment, animals were sedated (ketamine 40 mg/kg and acepromazine 6 mg/kg) and IOP measured at 0, 30, 60 and 120 min in both operated and contralateral control eye.

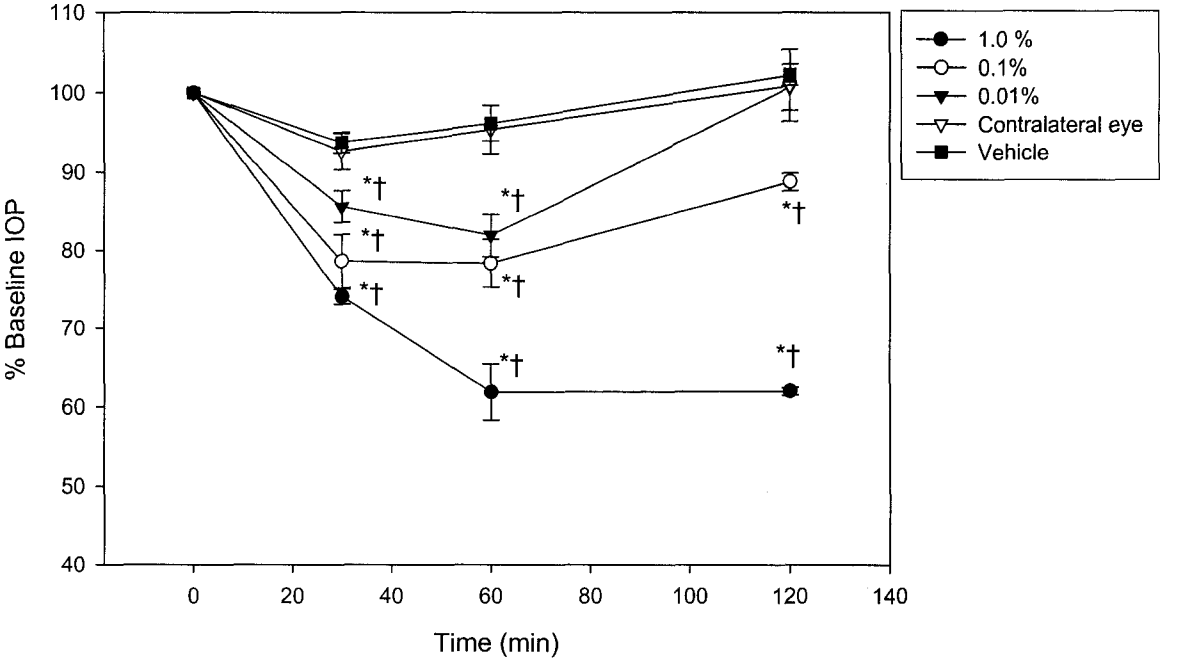

Figure 15: Dose response to topically applied O-1812 in surgically hypertensive eyes. The baseline IOP (t=0) was 16.0 ±0.9 mmHg. N = 10 rats/group.
* Significantly reduced when compared to baseline, $p < 0.05$.
† Significantly reduced when compared to vehicle, $p<0.05$.

Results

Effects of topically applied O-1812

At baseline (t=0), the IOP for the operated eye (OD) was 16.0 ±0.9 mm Hg and for the contralateral (non-operated) eye was 10.1 ± 0.5 mm Hg. Following a single topical application of O-1812 (1.0, 0.1 and 0.01%) or vehicle alone to the operated eye, the change in IOP (mm Hg) from baseline was measured for up to 120 min (Figure 15).

After 30 min, all concentrations of O-1812 significantly decreased IOP ($p < 0.001$) from 30 to 120 min. Maximal effect was observed at 60 min for all concentrations. At this time period, 1.0 % elicited a significantly greater IOP reduction than either 0.1 or 0.01% concentrations ($p<0.001$). At the end of the study period (120 min), only 1.0% and 0.1% treatment continued to maintain IOP reduction while for the 0.01% concentration IOP had already returned to baseline. Thus, magnitude and duration of effect was dose dependent. Tocrisolve™ (vehicle) alone had no effect on IOP ($p>0.08$). No crossover effects were seen in the contralateral eyes.

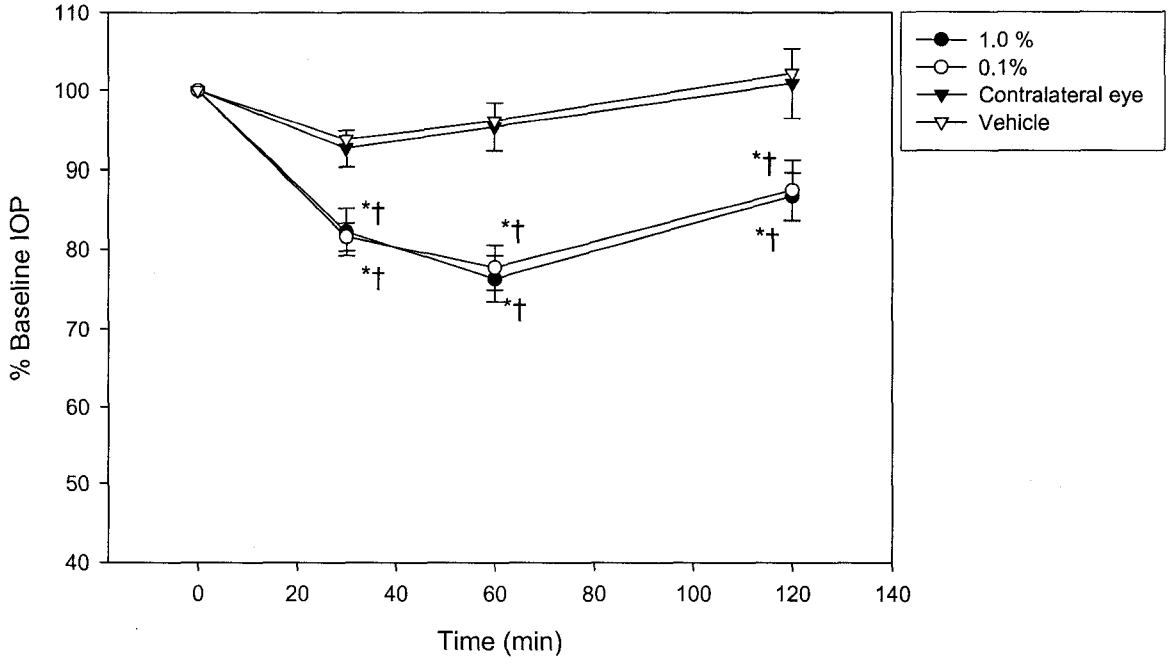

Figure 16: Dose response to topically applied O-2545 in surgically hypertensive eyes. The baseline IOP (t=0) was 16.0 ±0.9 mmHg. N = 7 rats/group.
* Significantly reduced when compared to baseline, $p < 0.05$.
† Significantly reduced when compared to vehicle, $p<0.05$.

Effects of topically applied O-2545

At baseline (t=0), the IOP for the operated eye (OD) was 16.0 ±0.9 mm Hg and for the contralateral (non-operated) eye was 10.1 ± 0.5 mm Hg. Following a single topical application of O-2545 (1.0 and 0.1%) or vehicle alone to the operated eye, the change in IOP (mm Hg) from baseline was measured for up to 120 min (Figure 16).

After 30 min, both concentrations of O-2545 significantly decreased IOP ($p < 0.03$, n=7) for up to 120 min. Maximal effect on IOP was observed 60 min after administration of O-2545. The IOP reduction was similar for both 1.0 and 0.1% concentrations throughout the study period. At the end of the study period (120 min), although IOP remained significantly reduced from baseline, both 1.0 and 0.1% treatments had already started to return toward baseline ($p < 0.05$, n=7). There was no effect of vehicle (PBS) on IOP as well as no effect in contralateral eye.

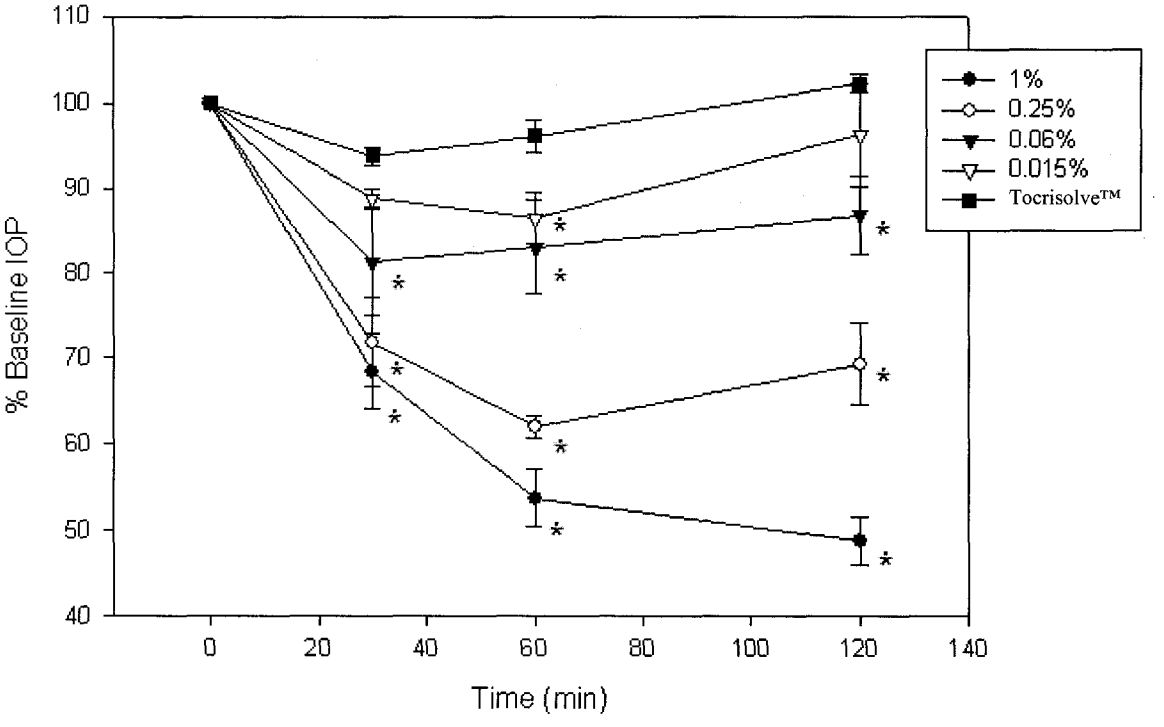

Figure 17: Topically applied WIN55212-2 in surgically hypertensive eyes. The baseline IOP (t=0) was 16.0 ±0.9 mmHg. N = 10 rats/group.
* Significantly reduced when compared to baseline, $p < 0.05$.

Effects of topically applied WIN55212-2

At baseline (t=0), the IOP for the operated eye (OD) was 16.0 ±0.9 mm Hg and for the contralateral (non-operated) eye was 10.1 ± 0.5 mm Hg. Following a single topical application of WIN55212-2 (1.0, 0.25, 0.06, and 0.015%) or vehicle alone to the operated eye, the change in IOP (mm Hg) from baseline was measured for up to 120 min (Figure 17).

By 30 min, all concentrations of WIN55212-2 significantly decreased IOP ($p < 0.001$) from 30 to 120 min, except for WIN55212-2 0.015% ($p = 0.59$, n=10). WIN55212-2 1.0, 0.25 and 0.06% also significantly reduced IOP compared to 0.015% concentration 30 min after administration ($p=0.03$). Maximal effect on IOP was observed 60 min after administration of WIN55212-2. At this time period, both 1.0 and 0.25% elicited a significantly greater IOP reduction than 0.06 and 0.015% concentrations ($p<0.001$). At the end of the study period (120 min), only 1.0% treatment continued to maintain IOP reduction while 0.25 and 0.06% concentrations had already started to return toward baseline. The magnitude and duration of effect was dose dependent. Vehicle alone had no significant effect on IOP ($p>0.08$).

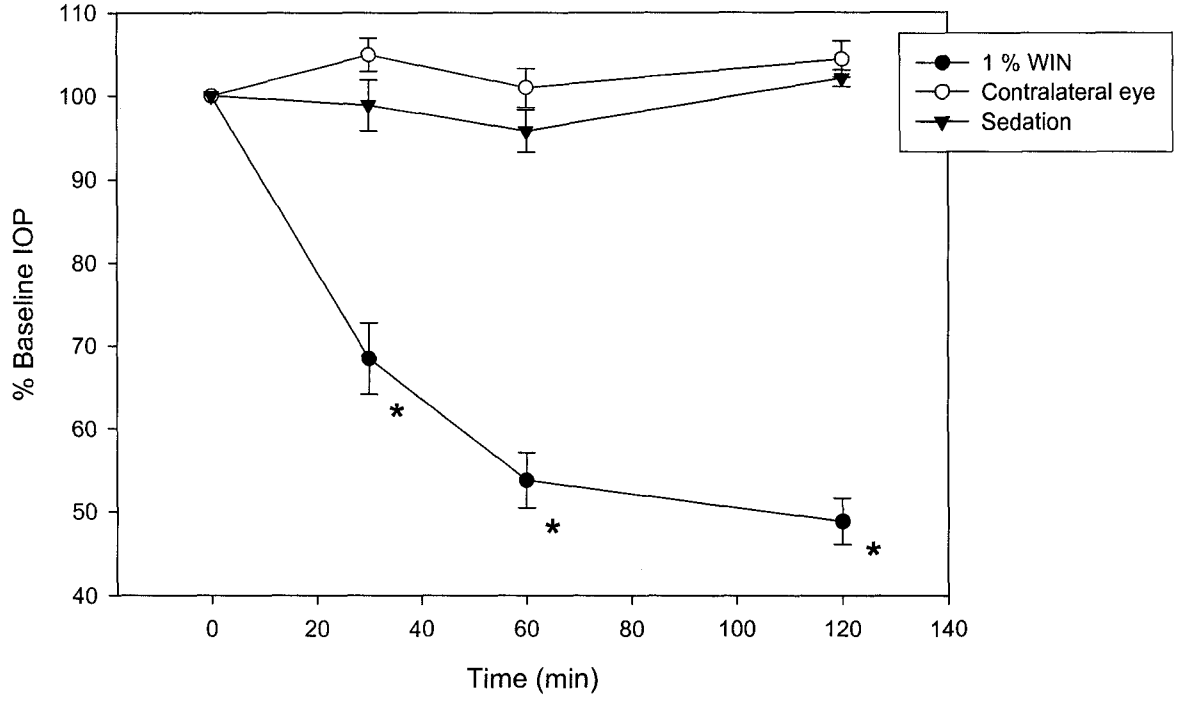

Figure 18: Topical administration of WIN55212-2 in the operated eye significantly reduced IOP, but had no effect in the contralateral eye.
Data presented as mean ± SEM.
* Significantly reduced when compared to baseline (t=0), $p < 0.001$, n=10.

Effect on contralateral eye

IOP changes in the contralateral control eye (OS) were recorded after treatment with WIN 55212-2, O-1812, O-2545 or vehicle. There was no apparent cross over effect in the contralateral control eye even at the highest concentration of WIN55212-2 1.0% (Figure 18). For example, IOP was reduced from 16.2 ±0.9 mmHg at baseline (t=0) to 9.0 ±0.5 mm Hg after 60 min in the treated eye. There was no concomitant change in the untreated eye ($p = 0.59$); i.e. IOP was 10.1 ± 0.5 mmHg at baseline and 10.4 ± 0.4 mmHg after 60 min (n=10). Also, after treatment with O-1812 or O-2545, IOP in the contralateral eye was unaffected (see Figure 15 and 16). Sedation, itself, did not significantly alter IOP in the hypertensive eye.

Effect of CB1 receptor antagonism on O-1812 or O-2545

Topical application of O-1812 1.0% alone reduced IOP by 26 % after 30 min and 38 % by 120 min (Figure 1). After pretreatment with CB1 antagonist, SR141716 (2mM), topical treatment with O-1812 had no effect on IOP at 30, 60 and 120 min, indicating significant antagonism of O-1812 by SR141716 (Figure 19).

When administered alone, O-2545 1.0% reduced IOP by 18 % after 30 min and 23 % by 120 min (Figure 2). Pretreatment with CB1 antagonist, SR141716 (2mM), did not reduce IOP within 30 min. However, a slight reduction of 9 ± 0.4% in IOP was observed 60 min after instillation of O-2545, which recovered to baseline value after 120 min. Topical administration of SR141716 (2mM to 0.02mM) alone had no effect on IOP (data not shown). No crossover effects were seen in the contralateral eye.

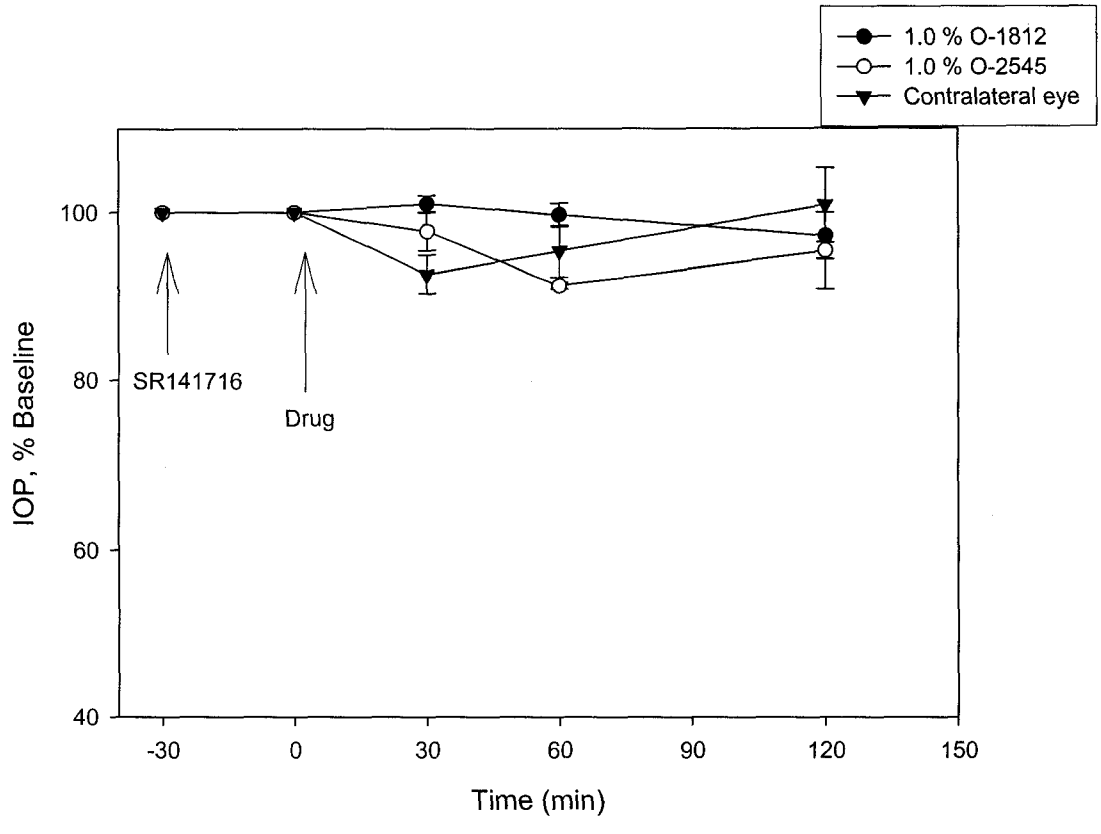

Figure 19: Effect on IOP of CB1 antagonist, SR141716, followed by O-1812 or O-2545. CB1 antagonist (2mM), SR141716, was applied topically 30 min (see arrow) prior to administration of each cannabinoid (t = 0 min). Data presented as mean ± SEM. N=6.

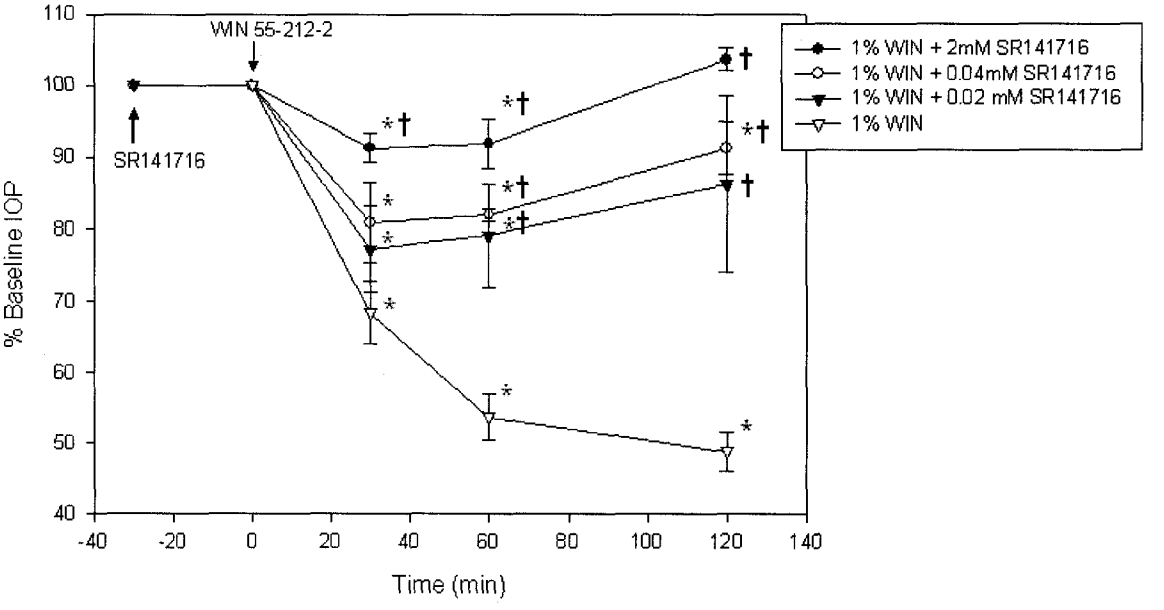

Figure 20: Effect on IOP of CB1 antagonist, SR141716, followed by WIN55212-2. CB1 antagonist, SR141716, was applied topically 30 min (see arrow) prior to administration of WIN55212-2 (t = 0 min). Data presented as mean ± SEM.
* Significantly reduced from baseline (t=0), $p<0.05$.
† Significantly greater than 1.0% WIN55212-2 alone treatment, $p<0.05$.

Effect of CB1 receptor antagonism on WIN 55212-2

Topical application of WIN55212-2 1.0% alone reduced IOP by 32 % after 30 min and 52% by 120 min (Figure 17). After pretreatment with CB1 antagonist, SR141716 (2mM), the maximal reduction of only 8.0±2.1% occurred at 30 min (n=3). This reduction was significantly less than WIN55212-2 alone ($p=0.009$), but significantly lower than baseline ($p=0.03$, n=3, Figure 20). IOP recovered to baseline within 120 min indicating significant antagonism of WIN55212-2 ($p<0.001$, compared to WIN55212-2 alone, n=3).

The effect of SR141716 on the response to WIN55212-2 was concentration dependent. After decreasing the concentration of SR141716 50 fold (0.04 mM), WIN55212-2 decreased IOP by 20% at 30 min and by 10% at 120 min ($p=0.15$, and $p<0.001$ respectively when compared to WIN55212-2 alone; $p=0.02$ and $p=0.05$ respectively when compared to baseline, n=3). After a 100 fold decrease (0.02 mM), WIN55212-2 maximally reduced IOP by 23% at 30 min and by 14% at 120 min ($p=0.29$, and $p=0.04$ respectively when compared to WIN55212-2 alone; $p=0.02$ and $p=0.06$ respectively when compared to baseline, n=3).

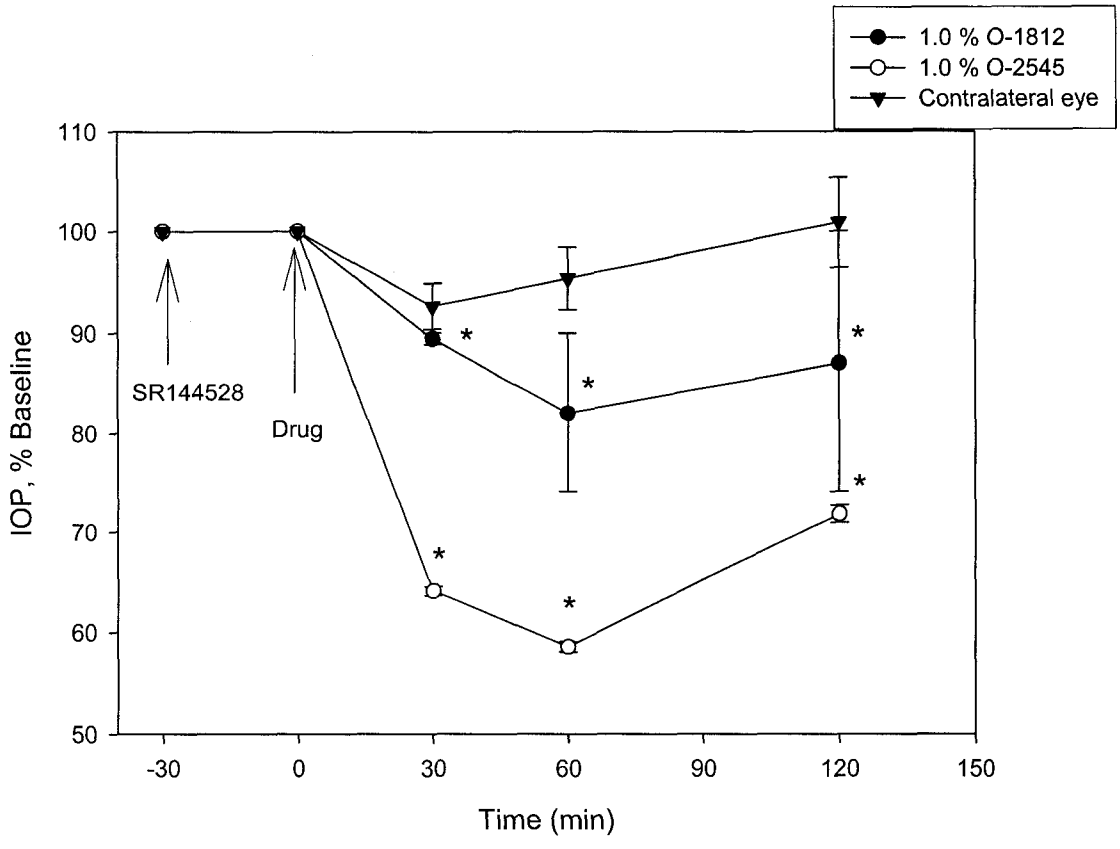

Figure 21: Effect of CB2 antagonist, SR 144528, followed by O-1812 or O-2545 on IOP was tested for 120 min.
CB2 antagonist was applied topically 30 min prior to administration of each cannabinoid. Data presented as mean ± SEM.
* Significantly reduced from baseline (t=0), $p<0.05$.

Effect of CB2 receptor antagonism on O-1812 or O-2545

Effects of pretreatment with CB2 antagonist, SR144528 (2mM), on IOP were studied after topical administration of O-1812 1.0%, or O-2545 1.0% (Figure 21, n=3). After pretreatment with SR144528, O-1812 1.0% reduced IOP by 11 ± 0.6% at 30 min ($p<0.0001$ when compared to O-1812 alone; $p=0.05$ when compared to baseline, n=3) and by 13 ± 1.3% mmHg after 120 min ($p=0.009$ when compared to O-1812 alone; $p=0.05$ when compared to baseline, n=3).

In the presence of SR144528, the IOP response to O-2545 1.0 % was decreased by 26 ± 0.4 % after 30 min ($p=0.15$ when compared to O-2545 alone; $p=0.03$ when compared to baseline, n=3) and by 28 ± 0.9% after 120 min ($p=0.005$ when compared to O-2545 alone; $p<0.0001$ when compared to baseline, n=3). Topical administration of SR144528 (2mM to 0.02mM) alone did not have any effect on IOP (data not shown).

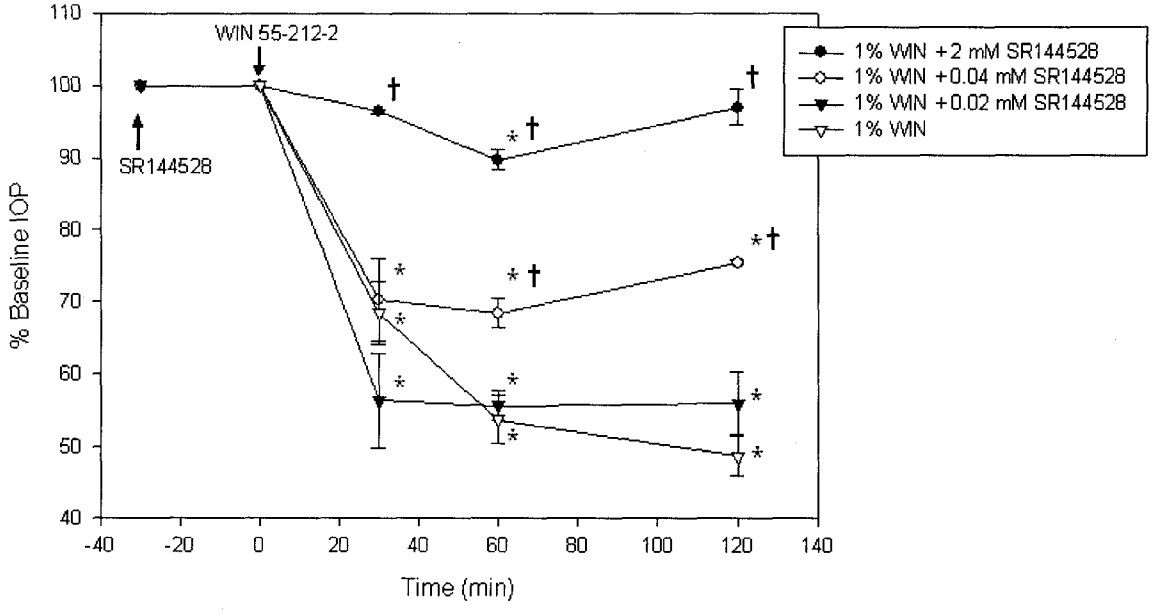

Figure 22: Inhibition of WIN55212-2 by the CB2 antagonist, SR 144528.
CB2 antagonist was applied topically 30 min prior to administration of WIN55212-2.
Data presented as mean ± SEM.
* Significantly different from baseline (t=0), $p<0.05$.
† Significantly different from WIN55212-2 1.0% alone treatment, $p<0.05$.

Effect of CB2 receptor antagonism on WIN 55212-2

Pretreatment with CB2 antagonist, SR144528 (0.02mM) did not significantly inhibit the effects of WIN55212-2 1.0% (Figure 22, n=3). Following SR144528 pretreatment, IOP was reduced from a baseline of 19.5±0.4 mmHg to 9.0±0.4 mmHg at 30 min ($p= 0.13$ when compared to WIN55212-2 alone; $p= 0.05$ when compared to baseline, n=3) and 9.0±0.1 mmHg at 120 min ($p=0.18$ when compared to WIN55212-2 alone; $p=0.03$ when compared to baseline, n=3).

When pretreated with higher doses of SR144528 (0.04mM or 2mM), the hypotensive effects of WIN55212-2 1.0% diminished. After doubling the concentration (0.04 mM), WIN55212-2 decreased IOP by 30% at 30 min and by 25% at 120 min ($p=0.80$, and $p= 0.01$ respectively when compared to WIN55212-2 alone; $p=0.02$ and $p=0.05$ respectively when compared to baseline, n=3). A further SR144528 increase (to 2mM), blocked the WIN55212-2 mediated IOP effect (for 30 and 120 min, $p=0.003$ and $p=0.001$ respectively when compared to WIN55212-2 alone; $p=0.11$ and $p=0.17$ respectively when compared to baseline, n=3).

Effects of WIN55212-2 on heart rate and blood pressure

Even at the highest concentration tested O-1812, O-2545 or WIN55212-2 (1.0%) had no effect on heart rate or blood pressure. Although, after administration of WIN 55212-2, heart rate apparently changed from 434 ±12 bpm at baseline to 414 ±10 bpm at

120 min, this change was not significant ($p=0.70$). Likewise, neither systolic nor diastolic blood pressure changed significantly ($p=1.00$, $p=0.67$, respectively compared to baseline).

Examination for ocular irritation

Slit lamp examination conducted at the end of each experiment revealed no signs of ocular toxicity attributable to vehicle, O-1812, O-2545, WIN55212-2, SR141716, or SR144528. No signs of inflammation, conjunctival chemosis/swelling, conjunctival discharge, aqueous fibrin/flare, loss of the pupillary light reflex, obscuration of iris structures, or corneal opacity were observed. In all treatment groups mild central corneal staining was noted consistently. However, similar corneal staining also occurred in the untreated control eyes. This finding was most likely a result of repeated applanation with the Goldmann tonometer rather than a drug- or vehicle-induced effect.

Corneal thickness

Confocal examinations of the cornea were performed at conclusion of each experiment. Treatment with O-1812, O-2545 or WIN 55212-2 did not change central corneal thickness as it remained at a baseline thickness of 320±25 μm.

Discussion

It has been over a decade since a new class of pharmacologic agents was introduced for the clinical management of glaucoma [25]. Currently available therapeutic approaches for the treatment of glaucoma include prostaglandin analogs, beta-adrenergic blockers, alpha-2 adrenergic agonists, carbonic anhydrase inhibitors and cholinergic agonists. Each of these has a well known profile of clinical response and adverse effects. Up to 50% of patients cannot be maintained on single drug therapy; most require use of two or even three drugs to control their IOP [97]. Even the first line agent for the treatment of glaucoma, timolol, as monotherapy controlled IOP in only 98 of 155 patients (63.2%) [34]. More recently, in the Ocular Hypertension Treatment Study, 40% of patients randomized to treatment required more than one medication to achieve the 20% reduction goal [35].

For over thirty years cannabinoids have been touted for their potential to decrease IOP [6]. However, cardiovascular and psychotropic effects complicate systemic administration [40, 41]. Numerous studies showed that systemic administration of cannabinoids produced an unwanted reduction in systolic and diastolic blood pressure, decreased heart rate and variable changes in pupil diameter [42-44]. Moreover, the associated psychotropic effects of these agents are well documented [45].

Topical administration of cannabinoids offers the theoretical advantage of providing desirable local ocular effects with minimal, if any, systemic side-effects. However, poor cannabinoid solubility has hindered preparation of a suitable topical

dosage form, resulting in an inadequate effect on IOP [142, 147]. Vehicles with potential for dissolving lipophilic cannabinoids include sesame oil [148, 149], mineral oil [148, 150], polyethylene glycol [141, 142], Tween 80 [148], and submicron aqueous emulsions [151]. While these agents have the ability to dissolve the lipophilic cannabinoids, they may also permit sufficient systemic absorption associated with undesirable systemic effects. For example, topical application of Δ 9 THC in a submicron emulsion significantly reduced IOP in rabbits, but systemic effects were also noted [151].

Tocrisolve™, a proprietary preparation, is a vehicle designed for lipophilic compounds, such as cannabinoids and vanilloids. Tocrisolve is composed of 1:4 soya oil and water and is emulsified with the block copolymer Pluronic F68. In the past we have used 2-hydroxypropyl-β-cyclodextrin as a vehicle to dissolve cannabinoids. Both 2-hydroxypropyl-β-cyclodextrin and Tocrisolve™ are good solvents for WIN55212-2. However, Tocrisolve™ not only dissolves up to 2% in WIN55212-2 compared to 1% in 2-hydroxypropyl-β-cyclodextrin but also does not require ethanol to promote solubility [90]. In this study, acute application of WIN55212-2, dissolved in Tocrisolve™ and applied topically, produced a significant reduction in IOP. Moreover, there was a notable lack of systemic effects, e.g. heart rate and blood pressure. In a recent study, 4 weeks of daily topical treatment of rats with WIN55212-2 in Tocrisolve™ did not cause local ocular toxicity, i.e. ocular inflammation, conjunctival chemosis/swelling, conjunctival discharge, aqueous flare or fibrin, diminished corneal light reflex, iris or corneal opacity, or corneal vascularization/staining [90]. This excellent tolerability profile supports the

potential use of these cannabinoid emulsions in the management of glaucoma in humans where long term treatment is needed.

In normotensive rabbits Song and Slowey speculated that WIN55212-2 exerts its ocular hypotensive effect in part through activation of CB1 receptors in the eye [16]. Studies designed to identify the distribution of cannabinoid receptor mRNA and protein in ocular tissues support this hypothesis. Using reverse transcriptase polymerase chain reaction technology, CB1 mRNA transcripts were expressed at significant amounts in the retina, ciliary body and iris, but CB2 mRNA transcripts were undetectable in human eyes [51]. Likewise, a similar distribution of CB1 mRNA, with an absence of CB2 mRNA, was found in eyes from ocular normotensive rats [13]. However, there is recent evidence for CB2 receptors on trabecular meshwork cells. Stimulation of these receptors with the CB2 agonist, JWH015, increased the aqueous humor outflow, thus contributing to their ocular hypotensive effect [53].

While the mechanism is unknown, pharmacological and histological studies support a direct role for CB receptors in ocular tissues of the human eye, including the ciliary epithelium, trabecular meshwork, Schlemm's canal, ciliary muscle, ciliary body vessels and retina [152]. Also, Sugrue suggests that cannabinoid receptors in the retina may convey neuroprotection to retinal ganglion cells [22]. In this study, topical application of O-1812 or O-2545 was compared to WIN55212-2 using ocular hypertensive rats. The effects of WIN55212-2 1.0% and O-1812 1.0% were comparable, while O-2545 1.0% was not as effective. Evidence from this study also indicates that the effect of O-1812,

O-2545 and WIN55212-2 is mediated primarily by CB1 receptor activation, congruent with the ability of the CB1 antagonist, SR141716, to inhibit the effects of these drugs (1%). When pretreated with SR141716 (2mM-0.02mM), the magnitude and duration of drug mediated hypotensive effect was severely diminished. In this study, the effect of SR141716 was noted as early as 30 min lasting for the entire study period. Although IOP was only measured for 2hr, Boyd and Fremming have demonstrated that when given orally, the duration of blockade by SR141716 is upto 8 hrs [153].

CB1 receptors are widely distributed in the eye. The local effect of cannabinoids at these receptors may be multifocal and remains to be fully elucidated. At the level of the ciliary body CB1 receptor-mediated control of IOP is likely due, at least partially, to decreased aqueous humor production [13, 147, 154, 155]. By contrast, CB1 receptors present in the trabecular meshwork may increase trabecular outflow [14, 52, 156]. Either site could be easily reached in therapeutic concentrations following topical application of CB1 agonists, as was done in the current study. Others have speculated that IOP effects could also be non-receptor mediated [16].

The role of CB2 receptors in IOP control has not been definitively determined. The Ki values of the CB2 antagonist (SR144528, Ki [CB2] = 0.6 nM, and Ki [CB1] = 437 nM) used in this study were determined in Chinese hamster ovary cells whose membranes express CB2 receptors [146]. The topical dose of SR144528 used in the current study was considerably higher (2.0mM-0.02mM) than the Ki [CB2] of 0.6 nM. Although the data indicate a preferential contribution of CB1 to CB2 receptors in the O-1812, O-

2545 and WIN 55212-2 mediated IOP reduction, the differences in receptor selectivity of SR144528 and SR141716 in vivo are much less than expected from the in vitro experiments. This may be related to a number of factors including the amount of antagonist delivered to the receptors or the residency of the antagonist on the receptor.

When administered topically in rabbits, the maximum concentration of timolol in the vitreous humor was a hundred-thousandth of that in the drop [157], indicative of the type of reduction in concentration that might be expected in our model. With a difference in vivo Ki, the blockade of O-1812 and WIN55212-2 observed at the higher concentrations of SR144528 could be due to administration of this agent in concentrations greatly exceeding its Ki [CB2] and approaching its Ki [CB1]. Thus, nonspecific binding to the CB1 receptor may have blunted the effect of O-1812 and WIN55212-2 in this setting. Although CB1 receptor effects are predominant, WIN55212-2 may also exhibit mixed agonist activity in some tissues suggesting that there may be some role for CB2 receptors in cannabinoid-mediated IOP control.

In this study we showed that topically applied novel cannabinoids, O-1812 and O-2545, effectively decrease IOP in a rat model of ocular hypertension. This effect on IOP was dose dependent and mediated predominantly through CB1 receptors. Equally important was a notable lack of systemic effects or local toxicity to ocular structures. Thus, a new class of medications, topically administered cannabinoids, to the current armamentarium offers the clinician more alternatives in the management of glaucoma.

CHAPTER VIII

O-COMPOUNDS CONFER NEUROPROTECTION VIA A CB1 MEDIATED PATHWAY

Introduction

The visual pathway begins at the photoreceptors. After light stimulation, a photoisomerization reaction in the rods and cones, initiates the visual cycle generating an electrical signal which traverses the visual pathway terminating in the visual cortex. While the electrical activity is transmitted to the visual cortex, the photoreceptors are replenished with trophic factors which originate from the visual cortex. Any break in the visual pathway leads to blindness due to either degeneration of the photoreceptors by termination of the trophic factors or the absence of electrical activity at the photoreceptors [158].

In glaucoma, risk factors such as elevated IOP, can contribute to the degeneration of photoreceptors especially the retinal ganglionic cells. It is postulated that NMDA channels are overstimulated as a result of an injury, for example hypoxia, which results in elevation of glutamate and subsequently intracellular Ca [2+] leading to excitotoxicity [26, 49]. NMDA receptors are expressed in both outer and inner retina, mostly RGC but limited in rods and bipolar cells [159, 160]. However, NMDA channels and glutamate are necessary for signal transduction especially in the bipolar cells [24, 161].

Naturally occurring delta-9-THC and synthetic cannabinoids, such as WIN 55212-2, protect neural cells from excitotoxicity as demonstrated in brain tissue and also against perinatal brain lesions [162-164]. Increase in reactive oxygen species as a result of excessive NMDA signalling can also be countered by delta-9-THC [165]. More recent finding suggests that NMDA mediated Ca [2+] entry into cells triggers cannabinoid signalling [166].

In the previous chapter the efficacy of newly synthesized O-compounds was established. Considering that the O-compounds are derivatives of delta-9-THC, they should also be neuroprotective. This is a pilot study to evaluate the neuroprotective effects of O-1812 and O-2545 in a rat NMDA model. We also tested the receptor specific neuroprotective effects by blocking the CB1 receptors with SR141716 and blocking CB2 receptors with SR144258. Additionally, the specific effect of cannabinoids on Ca (2+) influx was examined.

Experimental Design

To induce retinal damage, male Sprague Dawley rats weighing ~ 250 gm, 50 to 55 days old (Harlan, Indianapolis, IN) underwent intravitreal NMDA injections. To determine the neuroprotective effects of the cannabinoids, ERG responses were compared between NMDA only treatment and NMDA + (O-1812 or O-2545) treatment. After baseline ERG measurements, animals were injected with NMDA. ERG was

measured after 1 and 2 wks. For the NMDA control group, 2 µl of 10mM NMDA was injected into the vitreous. For cannabinoid treated groups, 20 µl of either O-1812 1.0% or O-2545 1.0% was administered topically (b.i.d) for 3 days prior and for 2 days after the NMDA injection. To determine receptor specificity, 10 µl of either CB1 antagonist (SR141716, 2 mM) or CB2 antagonist (SR144258, 2 mM) was injected intravitreally followed 30 min later by NMDA. For these experiments, rats were also pre-treated with O-1812 or O-2545 for 3 days prior and for 2 days after the NMDA and CB (1 or 2) receptor antagonist injection.

ERG measurements were made scotopically using calibrated bright flashes to stimulate the photoreceptors. In the dark cycle, the photoreceptors are in a depolarized state, but when stimulated the photoreceptors repolarize and generate a characteristic wave pattern known as the a-wave. With NMDA toxicity, the photoreceptors along with the a-wave response are greatly affected [167]. It is therefore sound to focus on the a-wave response initially to determine the extent of neuroprotection in the photoreceptors. The contralateral normal eye served as an age matched negative control, e.g. response in a normal, untreated eye. Differences in amplitude of the a-waves between eyes treated with NMDA only, the positive control, and NMDA + O-compounds were calculated and analyzed. All data is reported as a percentage of the baseline.

At the end of 2 wks, animals were sacrificed and eyes enucleated for histology. Retinas were harvested by bisecting the eyes peri-limbally and removing the anterior segment. The remaining posterior segment cup was flattened with 4 radial cuts after

which the retinal layer was carefully dissected and stained with Hematoxylin and Eosin. For positive identification of RGC, retinal flat mounts were compared with cross-sections for cell shape, placement and distribution. Cell counts were performed by knowledgeable masked observers. For each retina, at least 2 microscopic fields of 50 μm^2 were captured per quadrant at predetermined distance from the optic cup (200 μm). The total retinal area was approximately 500 μm^2. Retinal cell counts were averaged for each field and comparisons made between O-compound treated, contralateral control and positive control NMDA treated eyes. Retinal cell counts are expressed as cells per 50 $\mu m^2 \pm SD$.

Results

NMDA induced excitotoxicity is prevented by novel cannabinoids

NMDA and endocannabinoids altered retinal function (Figure 23). Damage induced by NMDA was severe and reduced a-wave amplitude by 36±16 % ($p<0.001$, n=6) from baseline in one week. Co-administration of NMDA with O-2545 resulted in a 41±17 % ($p<0.001$, n=6) loss a-wave amplitude, similar to that of NMDA alone after one week. However, only O-1812 effectively mitigated the toxic effect of NMDA in one week, with only a 19±13 % loss in a-wave amplitude, which while significantly less than baseline ($p<0.01$, n=6), was also a significantly smaller decrease than NMDA alone ($p=0.05$, n=6).

After 2 wks, NMDA induced damaged continued to progress to 46±9 % a-wave amplitude loss ($p<0.001$, n=6), but cannabinoid treatment resulted in improved retinal function. Lipid soluble O-1812 was the most effective endocannabinoid as a-wave amplitude was only 7±12 % less than baseline after two weeks, which was not significantly less than baseline ($p=0.08$, n=6). Water soluble O-2545 also countered the effects of NMDA but retinal function was significantly less than baseline (31±13 %, $p<0.05$, n=6). Thus, it was not as effective as O-1812.

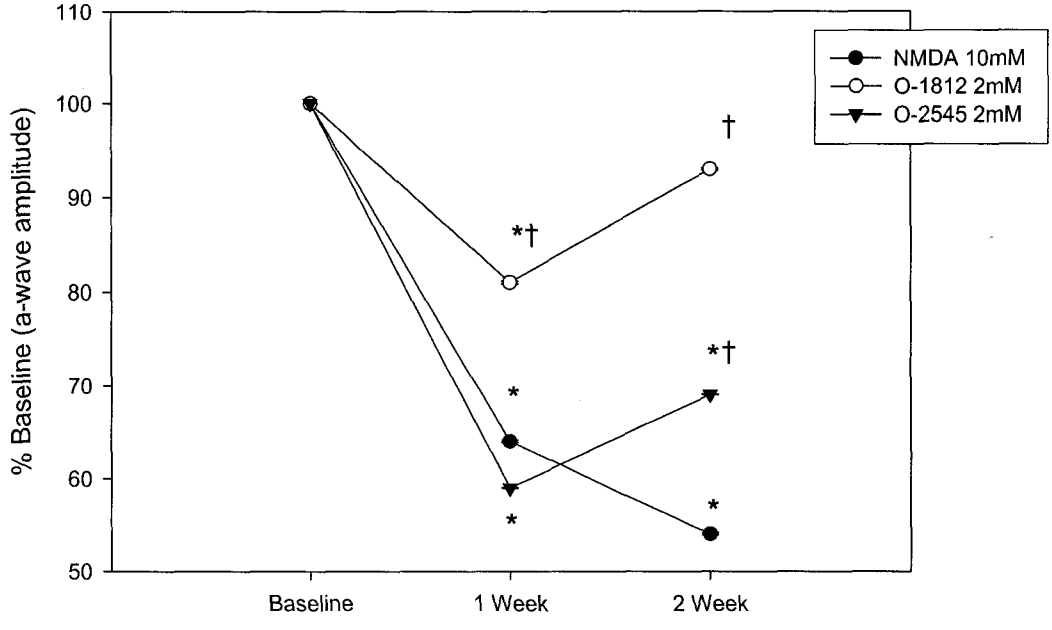

Figure 23: Topical treatment with O-1812 or O-2545 after intravitreal injection of NMDA.
Retinal function was measured with electroretinography (ERG) one and two weeks after NMDA injection. Alterations in retinal response as a result of treatments were assessed by the a-wave amplitude and expressed as percent of baseline.
* Significantly reduced from baseline, $p<0.001$
† Significantly greater than NMDA alone, $p<0.05$

Table 5: Retinal function was measured with electroretinography (ERG). Alterations in retinal response as a result of treatments were assessed two weeks after NMDA injection by quantitating a-wave amplitude. Results are expressed as percent loss from baseline.

	Drug alone	+ SR141716	+ SR144258	N
Two Week	*% loss ± SEM*	*% loss ± SEM*	*% loss ± SEM*	
NMDA	46±9*			6
O-1812	7±12†	45±17*	7±10†	6
O-2545	31±13*†‡	32±8*†	34±7*†‡	6
	* Significantly less than baseline, $p<0.001$			
	† Significantly less than NMDA alone treatment, $p<0.001$			
	‡ Significantly greater than O-1812 alone treatment, $p<0.03$			

Neuroprotective effect in the presence of CB1 receptor antagonist

To determine specific CB receptor involvement in neuroprotection, CB1 antagonist SR-141716 (2mM) was injected intravitreally while either O-1812 or O-2545 (2mM or 1.0%) was administered topically (Table 5). After two weeks, the CB1 antagonist attenuated the effects of O-1812 as the a-wave amplitude loss was 45±17 % which was significantly less than baseline and O-1812 alone ($p<0.001$, n=6). SR-141716 did not have an effect on O-2545 action as a-wave amplitude loss was 32±8%, similar to that of O-2545 alone treatment (31±13%).

Neuroprotective effect in the presence of CB2 receptor antagonist

CB2 receptor antagonist, SR-144258 (2mM), was injected intravitreally to determine the role of CB2 receptors in neuroprotection (Table 5). Either O-1812 or O-2545 (2mM or 1.0%) was administered topically. After two weeks, SR-144258 did not have any effect on the activity of O-1812 as a-wave loss was 7±10 % which was similar to O-1812 alone treatment (7±12 %). SR-144258 also did not effect on O-2545 action as a-wave amplitude loss was 32±8%, similar to that of O-2545 alone treatment (31±13%).

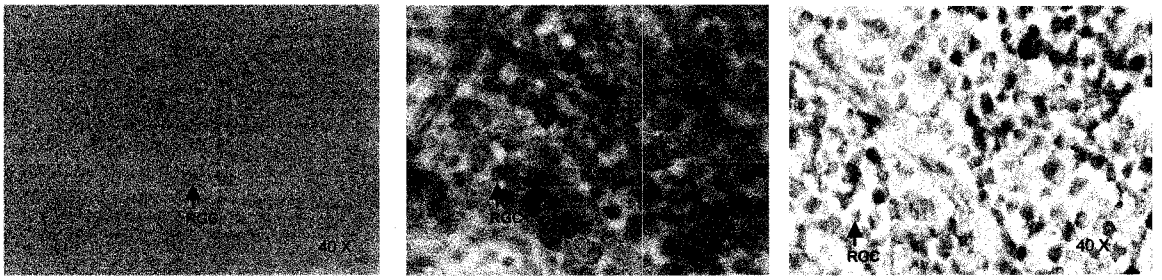

Figure 24: Flat mounted retinas stained with H&E.
Retinas were harvested at terminal point of the experiment (two weeks).

Endocannabinoids preserve retinal cell density

Representative retinas from each group were analyzed for morphological changes (Figure 24). The analysis of RGC count was limited to the retinal flat mounts because the difficulty in obtaining consistent and comparable results with cross-sections. Also, the retinal damage was not localized to a particular area; therefore, using cross sections to assess morphological changes in the retina would have been less representative than the retinal flat mounts. Retinal ganglionic cells were positively identified in the flat mounts by comparing attributes (e.g. cell shape, distribution) with cross sections. After NMDA injection RGC cell density was significantly reduced to 46.2±22.3 compared to 101±5.6 in the contralateral control ($p<0.001$). After treatment with O-1812 or O-2545 RGC cell density was 76.4±12.1 and 67.4±6.5 respectively. Cell density was significantly greater in the cannabinoid treated groups compared with NMDA alone ($p=0.001$). Also, both cannabinoids were equally efficacious as both had a similar effect to prevent retinal cell loss ($p=0.58$).

O-compounds inhibit Ca [2+] influx

In this pilot experiment the role of Ca [2+] influx as the mechanism of action for neuroprotection was investigated. Experiments were performed on transformed rat retinal ganglionic cells (RGC-5) with NMDA as a positive control. After NMDA (1mM) treatment, cells were incubated with 10 uM of either O-1812 or O-2545 (Table 6). After NMDA only treatment, the calcium signal after 60 min was 445% higher than baseline.

With O-1812 treatment, Ca [2+] influx was much less than that of NMDA alone after 60 min as the calcium signal was increased by 14%. After O-2545 treatment, the calcium signal was increased by 303% compared to baseline.

Discussion

Neuroprotection is an important strategy to combat the irreversible loss of retinal ganglionic cells and eventually the optic nerve. While risk factors such as elevated IOP, ischemia and genetics play an important role in the progression of retinal neuropathy, they all share a common characteristic i.e. loss of loss of retinal cells especially the ganglionic cells [26]. While the specific trigger is unknown, experimental evidence suggests that retinal cells undergo apoptosis [26, 140, 168-170]. Therapies that address both neuroprotection and reduce risk factors offer the greatest potential to combat retinal degenerative disorders.

In glaucoma, the most likely mechanism for retinal cell apoptosis is by overstimulation of sodium channels, namely NMDA, that increase basal glutamate levels leading to excitotoxicity [23, 26]. In addition to NMDA channels, metabotropic receptors, such as mGlu, can also elevate glutamate levels [171-173]. Excess glutamate via the NMDA receptor increases intracellular Ca [2+] and targets the cell for apoptosis [23, 26]. NMDA receptors, therefore, represent a therapeutic target for neuroprotection. In fact, NMDA antagonists, such as MK801 and memantine, have shown to be neuroprotective in rat ocular hypertensive models [119, 171, 174]. Memantine failed to show neuroprotection in clinical trials.

Table 6: Percent increase in Ca [2+] influx in RGC-5 cells as a result of NMDA, NMDA + O-1812 or NMDA + O-2545 treatment

	NMDA (1mM)	O-1812 (10 uM)	O-2545 (10uM)	Untreated
Baseline	0	0	0	0
15 min	3	0	29	8
30 min	11	2	102	13
60 min	346	140	203	150
A23187	382	128	285	351

It is desirable that new glaucoma drugs target the reduction of the risk factors, such as elevated IOP, as well as preserve retinal cells from apoptosis. In chapter 7, the IOP reduction potential of the synthetic O-compounds was demonstrated. The lipophilic O-1812 reduced IOP to a greater extent than the hydrophilic O-2545. Reduction of intraocular pressure, while being a sound strategy for glaucoma treatment, does not treat the underlying cause of glaucoma, retinal degeneration. Currently available drugs for glaucoma only reduce IOP. Data from this study suggest that O-compounds are neuroprotective, i.e. they retain the density and functionality of the photoreceptor and ganglionic cells. In electroretinographic evaluation two weeks after NMDA treatment, severe ERG a-wave deficits were observed. Concomitant treatment with either O-1812 or O-2545 reduced the a-wave amplitude loss, thereby preserving photoreceptor function. This effect was significantly better after O-1812 treatment than O-2545. These findings also corroborated with histology obtained from retinal flat mounts. Retinas from a subsection of animals were removed and flat mounted and ganglionic cell density determined two weeks after the induction of retinal damage by NMDA or after treatment with NMDA + either O-1812 or O-2545. Both O-compounds mitigated the NMDA induced ganglionic cell loss; however, O-1812 was more efficacious.

In normotensive rabbits Song and Slowey speculated that cannabinoids such as WIN 55212-2 exerted ocular effects in part through activation of CB1 receptors in the eye [16]. Studies designed to identify the distribution of cannabinoid receptor mRNA and protein in ocular tissues support this hypothesis. Using reverse transcriptase polymerase chain reaction technology, CB1 mRNA transcripts were expressed at significant amounts

in the retina, ciliary body and iris but CB2 mRNA transcripts were undetectable in human eyes [51]. Likewise, a similar distribution of CB1 mRNA, with an absence of CB2 mRNA, was found in eyes from ocular normotensive rats [13]. CB1 receptors are widely distributed in the eye. The local effect of cannabinoids at these receptors may be multifocal and remains to be fully elucidated. While at the level of the ciliary body CB1 receptor-mediated control of IOP is likely due, at least partially, to decreased aqueous production, their effect on neuroprotection is not fully understood [13, 147, 154, 155]. In our experiments, the CB antagonists affected IOP reduction as well as neuroprotection. The CB1 antagonist blocked the neuroprotective action of O-1812, a potent CB1 receptor agonist, but had no effect on the action of O-2545, a mixed CB receptor agonist. The CB2 receptor antagonist did not have an effect on the actions of either O-compound. These results suggest that the neuroprotective effect could easily be mediated by the CB1 receptor, which is similar to the proposed mechanism of IOP reduction. Kim et. al., have also reported CB1 activation as a possible mechanism of neuroprotection against NMDA toxicity [175].

Although both O-compounds exhibit similar affinities for the CB1 receptor, for O-1812 (CB1 K_i = 3.4±0.5 nM, CB2 K_i = 3870±235 nM)[79] and for O-2545 (CB1 K_i = 1.5±0.2 nM, CB2 K_i = 0.32±0.02) [87], the differences in effect can be attributed to variabilities in ocular penetration. Receptor activation is a function of drug availability at the target site. It well known that lipid soluble compounds have a constant and higher bioavailability in the ocular environment than water soluble compounds [176]. In chapter 6, the ocular penetration of O-1812 was shown to be greater than O-2545. This variability

in ocular penetration affects both neuroprotection and IOP reduction potential. After a single dose of O-1812, a 2 nM concentration was maintained in the vitreous chamber for 120 minutes, while O-2545 remained for only 80 minutes. Pretreatment with O-1812 for 3 days prior and two days after NMDA injection, a concentration of at least 2 nM would have been maintained in the vitreous chamber to mitigate the effects of NMDA. It is, therefore, likely that a pharmacologic concentration might not have been achieved with the hydrophilic drug to induce significant neuroprotection.

After NMDA injection, toxic effects can be observed in the retina in as little as 3 hours with a peak effect at 12 hours [177]. In order for a drug to have an effect, a pharmacological concentration must be achieved at the retina during the initial stages of NMDA toxicity, i.e. within 3 hours. With topical (b.i.d) pretreatment for 3 days and an additional 2 days after the NMDA injection, O-compounds were present in sufficient concentration at the retina to mitigate the toxic effects of NMDA. When ERG measurements were obtained after 1 week, the NMDA treated group showed a significant loss in ERG function which continued to decline. In the presence of both O-compounds, although there was some loss of function at 1 week, it improved over the period of 2 weeks. This phenomenon could be due to the toxic shock suffered by the retinal cells which could disrupt, fragment or ablate retinal cells [178]. Retinal function could also be compromised due to the disruption of the osmotic balance after intravitreal injections [179]. It is important to note that even with a limited dosing regimen, substantial neuroprotective effects were observed with either O-compound; however, O-1812 was significantly more effective.

In an initial attempt to determine the mechanism of action, the role of intracellular Ca [2+] influx was considered. In this pilot experiment, using isolated retinal ganglionic cells, both O-compounds reduced the influx of Ca [2+]. This needs to be explored further to identify downstream signalling events leading to apoptosis. In conclusion, O-compounds are a new class of drugs that are efficacious ocular hypotensive and neuroprotective agents. They can reduce the associated risk factors such as elevated IOP and also address the underlying pathology of glaucoma by protecting retinal cells from excitotoxic insults.

CHAPTER IX

EFFECT OF COMBINING CANNABINOIDS WITH COMMONLY PRESCRIBED GLAUCOMA MEDICATIONS ON INTRAOCULAR PRESSURE IN A RAT OCULAR HYPERTENSIVE MODEL

Introduction

Despite the currently available therapeutic approaches for the treatment of glaucoma, up to 50% of patients cannot be maintained on single drug therapy; most require use of two or even three drugs to control their IOP [97]. Even the first line agent for the treatment of glaucoma, timolol, as monotherapy controlled IOP in only 98 of 155 patients (63.2%) [34]. More recently, in the Ocular Hypertension Treatment Study, 40% of patients randomized to treatment required more than one medication to achieve the 20% reduction goal [35].

Commonly used classes of agents include β-adrenergic antagonists e.g. timolol, prostaglandin analogs e.g. latanoprost and α-adrenergic agonists e.g. brimonidine [98]. Timolol lowers IOP by decreasing aqueous humor production while brimonidine, in addition to decreasing aqueous humor production, has the added benefit of increasing uveoscleral outflow [99]. These two drugs when used concomitantly have an additive effect on IOP [180-183]. Other popular agents are carbonic anhydrase inhibitors e.g. dorzolamide and prostaglandin analogs e.g. latanoprost. Dorzolamide reduces aqueous humor

formation while latanoprost increases uveoscleral outflow [100, 101]. Endocannabinoid analogs are a new class of agents that are efficacious ocular anti-hypertensives. These analogs exert their effects by activating the cannabinoid receptor system (CB1/CB2). Their exact mechanism of action is largely unknown. The purpose of this study is to compare efficacy of timolol, dorzolamide, travoprost or brimonidine as monotherapy or in combination with endocannabinoid analogs, O-1812 or O-2545, in a rat glaucoma model.

Experimental design

Sprague Dawley rats were randomly assigned to receive 20 µl of either timolol 0.5%, O-1812 1.0%, O-2545 1.0%, brimonidine 0.2%, dorzolamide 2%, travoprost 0.004% alone or in combination, applied topically to the right eye. For combination therapy, timolol 0.5%, dorzolamide 2.0% or brimonidine 0.1% were applied topically followed 10 minutes later with either O-1812 1.0% or O-2545 1.0%. The contralateral eye (OS) served as the untreated control. At the beginning of each experiment, animals were sedated (ketamine 40 mg/kg and acepromazine 6 mg/kg) and baseline IOP, heart rate (HR), blood pressure (BP) measurements (t=0) were obtained. IOP measurements were obtained 30, 60 and 120 min after drug administration. HR and BP were also measured at baseline and towards the end of the experiment. Before and at the conclusion of each experiment, all eyes were examined and graded by slit lamp for signs of ocular irritation.

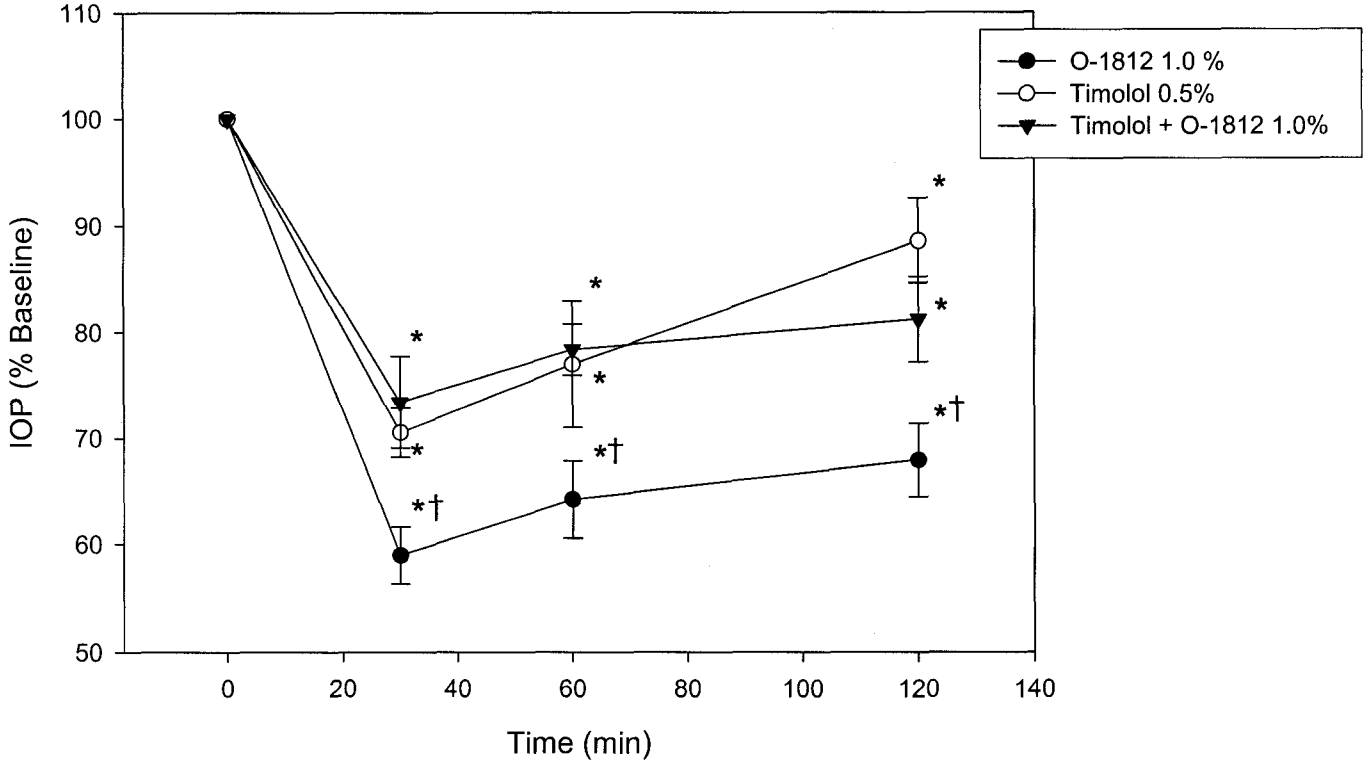

Figure 25: O-1812 1.0% and timolol 0.5% were applied alone or in combination and IOP recorded for 120 min.
Data are normalized to baseline measurements and presented as mean ± SEM.
* Significantly reduced compared to baseline (time = 0 min) ($p<0.05$),
† Significantly reduced compared to timolol and combination ($p<0.05$)

Results

Combination of O-1812 and timolol

Timolol and O-1812 were administered alone or in combination to evaluate their effect on IOP (Figure 25). At the beginning of the experiment, baseline IOP measurements were obtained after which O-1812 1.0% or timolol 0.5% was applied (time = 0 min, baseline, n=4 per group). For combination treatment, timolol 0.5% was applied first followed 10 minutes later with O-1812 1.0% at baseline (t=0).

After 30 min, a single dose of timolol reduced IOP from 23±0.6 mmHg at baseline to 16±0.8 mmHg, O-1812 reduced IOP from a baseline of 27±1.2 mmHg to 17±0.9 mmHg while the combination resulted in an IOP of 19±0.6 mmHg from a baseline of 27±1.0 mmHg (n=4). After this time point, no further reduction in IOP was observed. After 60 min, IOP remained significantly reduced in all treatment groups when compared to the baseline. However, IOP reduction achieved with O-1812 was significantly greater than that of timolol alone or the combination.

The experiment was terminated 120 min after drug administration. A final IOP measurement of 20±0.4 mmHg was obtained in the timolol 0.5% treatment group, 18±0.4 mmHg for O-1812 1.0% and 21±0.5 mmHg for the combination. All treatments significantly reduced IOP compared to baseline ($p<0.05$, n=4 per group). O-1812 was most efficacious compared with timolol or the fixed dose combination, no additive or synergistic effects were observed after combination therapy.

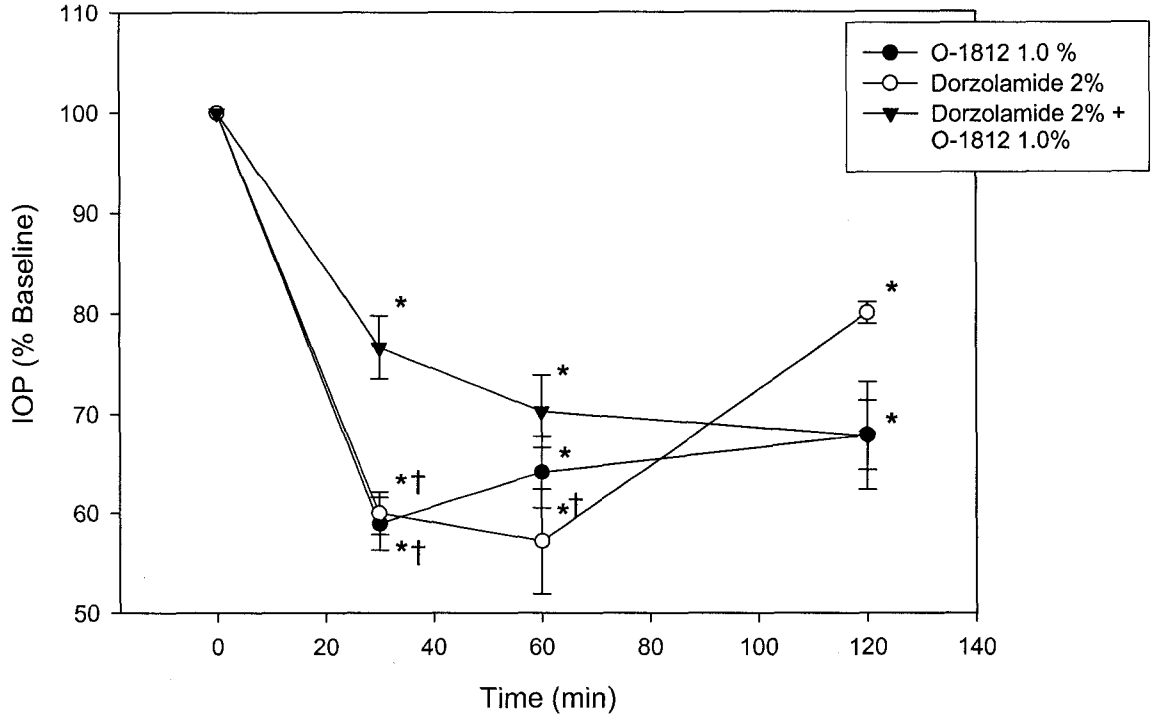

Figure 26: O-1812 1.0% and dorzolamide 2% were applied alone or in combination and IOP recorded for 120 min.
Data are normalized to baseline measurements and presented as mean ± SEM.
* Significantly reduced compared to baseline (time = 0 min) ($p<0.05$)
† Significantly reduced than the combination ($p<0.05$)

Combination of O-1812 and dorzolamide

The effects of dorzolamide and O-1812 on IOP were evaluated when applied alone or in combination (Figure 26). At the beginning of the experiment, baseline IOP measurements were obtained after which O-1812 1.0% or dorzolamide 2.0% was applied (n=4 per group). For the combination treatment, dorzolamide 2.0% was applied first followed 10 minutes later by O-1812 1.0% at baseline (t=0).

After 30 min, dorzolamide alone reduced IOP from a baseline of 23±0.3 mmHg to 14±0.5 mmHg, O-1812 reduced IOP from a baseline of 27±1.2 mmHg to 17±0.9 mmHg while the combination treatment reduced IOP from a baseline of 24±1.1 mmHg to 19±1.2 mmHg (n=4). At this time point, combination of O-1812 and dorzolamide did not produce an additive or synergistic reduction in IOP.

After 60 min, dorzolamide had the greatest effect with an IOP of 13±1.2 mmHg compared to 17±0.4 mmHg for O-1812 and 17±0.5 mmHg for the combination. Although significantly reduced from baseline, the effect of dorzolamide was not sustained past 60 min. After 120 min, the IOP reduction achieved with O-1812 alone and the combination treatment was of a longer duration and greater than dorzolamide treatment alone.

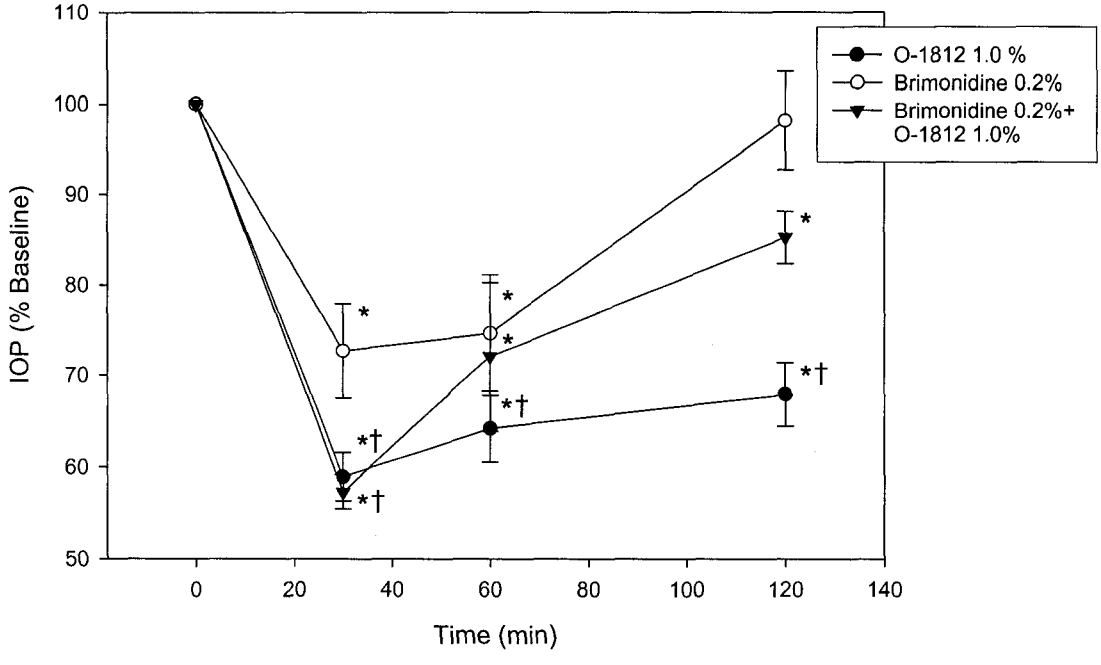

Figure 27: O-1812 1.0% and brimonidine 0.2% were applied alone or in combination and IOP recorded for 120 min.
Data are normalized to baseline measurements and presented as mean ± SEM.
* Significantly reduced compared to baseline (time = 0 min) ($p<0.05$)
† Significantly reduced than brimonidine alone ($p<0.05$)

Combination of O-1812 and brimonidine

Brimonidine and O-1812 were administered alone or in a fixed dose combination to evaluate their effect on IOP (Figure 27). At the beginning of the experiment, baseline IOP measurements were obtained after which O-1812 1.0% or brimonidine 0.2% were applied (n=4 per group). For combination treatment, brimonidine 0.2% was applied first followed 10 minutes later with O-1812 1.0% at baseline (t=0).

After 30 min, a single dose of brimonidine reduced IOP from a baseline of 23±1.5 mmHg to 16±1.1 mmHg, O-1812 reduced IOP from a baseline of 27±1.2 mmHg to 17±0.9 mmHg while the combination treatment reduced IOP from a baseline of 25±0.8 mmHg to 14±0.7 mmHg (n=4). At this time point, the fixed dose combination significantly reduced IOP compared to brimonidine alone but was not different than O-1812 alone.

In all treatment groups, maximal effect was achieved 30 min after drug administration after which IOP reverted towards baseline. At the end of the experiment (120 min), IOP in the O-1812 group (18±0.4 mmHg) and in the combination group (21±1.2 mmHg) was still less than baseline. However, the brimonidine treatment group returned to 22±0.5 mmHg, which was not significantly different from baseline (23±1.5 mmHg).

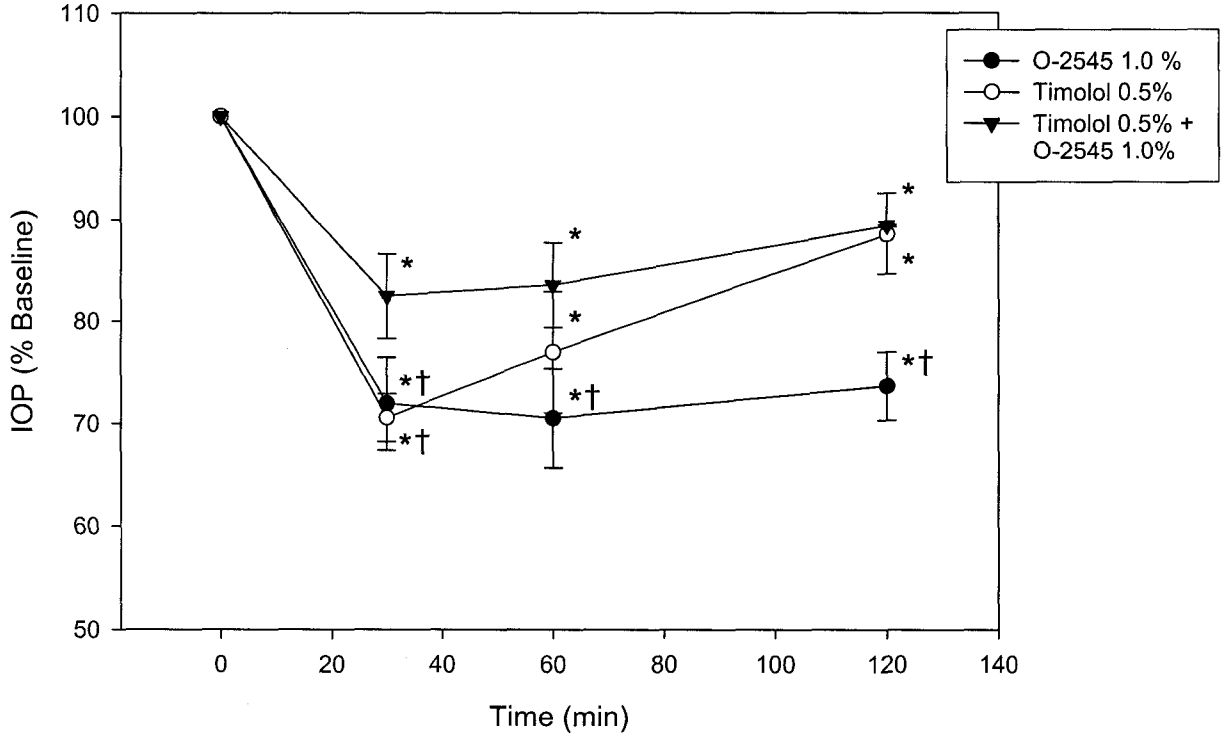

Figure 28: O-2545 1.0% and timolol 0.5% were applied alone or in combination and IOP recorded for 120 min.
Data are normalized to baseline measurements and presented as mean ± SEM.
* Significantly reduced compared to baseline (time = 0 min) ($p<0.05$)
† Significantly reduced than combination ($p<0.05$)

Combination of O-2545 and timolol

The efficacy of timolol and O-2545 when administered alone or in a fixed dose combination was evaluated (Figure 28). At the beginning of the experiment, baseline IOP measurements were obtained after which O-2545 1.0% or timolol 0.5% were applied (n=4 per group). For the combination treatment timolol 0.5% was applied first followed 10 minutes later with O-2545 1.0% at baseline (time = 0 min, n=4 per group).

After 30 min, a single dose of timolol reduced IOP from 23±0.6 mmHg at baseline to 16±0.8 mmHg, O-2545 reduced IOP from 25±1.4 mmHg to 18±0.4 mmHg while the combination resulted in a decline in IOP from 28±0.3 mmHg to 23±1.0 mmHg (n=4). At this time point, the combination of O-2545 and timolol was least efficacious compared with O-2545 or timolol alone.

Maximal efficacy for timolol and the combination was achieved after 30 min, and after 60 min with O-2545. At the end of the experiment (120 min) IOP remained decreased in the O-2545 group (18±0.5 mmHg) but tended to return toward baseline in the timolol (20±0.4 mmHg) and the combination group (25±0.8 mmHg).

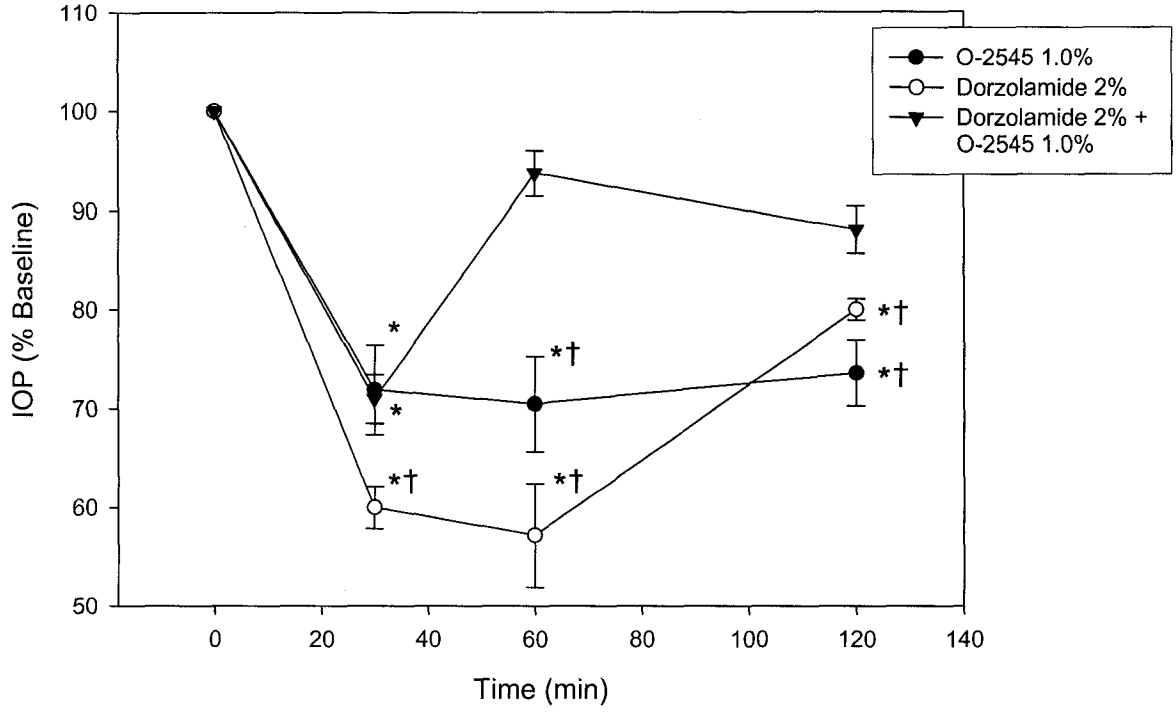

Figure 29: O-2545 1.0% and dorzolamide 2% were applied alone or in combination and IOP recorded for 120 min.
Data are normalized to baseline measurements and presented as mean ± SEM.
* Significantly reduced compared to baseline (time = 0 min) ($p<0.05$)
† Significantly reduced than combination ($p<0.05$)

Combination of O-2545 with dorzolamide

The effects of dorzolamide and O-2545 on IOP were evaluated when applied alone or in combination (Figure 29). At the beginning of the experiment, baseline IOP measurements were obtained after which O-2545 1.0% or dorzolamide 2.0% were applied (n=4 per group). For the combination treatment, dorzolamide 2.0% was applied first followed 10 minutes later with O-2545 1.0% (n=4).

After 30 min, a single dose of dorzolamide reduced IOP to 14±0.5 mmHg from a baseline of 23±0.3 mmHg, O-2545 reduced IOP to 18±0.4 mmHg from a baseline of 25±1.4 mmHg while the combination treatment reduced IOP to 20±0.6 mmHg from a baseline of 28±0.6 mmHg (n=4). At this time point, dorzolamide was more efficacious than either O-2545 alone or the combination ($p<0.05$, n=4).

Dorzolamide treatment reduced IOP to 13±1.2 mmHg after 60 min and O-2545 treatment reduced IOP to 18±1.0 mmHg, while their combined effect on IOP was only 26±0.4 mmHg. The combination treatment was least efficacious as IOP was not significantly different than baseline after 60 and 120 min after administration. A final IOP of 18±0.5 mmHg for O-2545 and 19±0.3 mmHg for dorzolamide treatment were recorded.

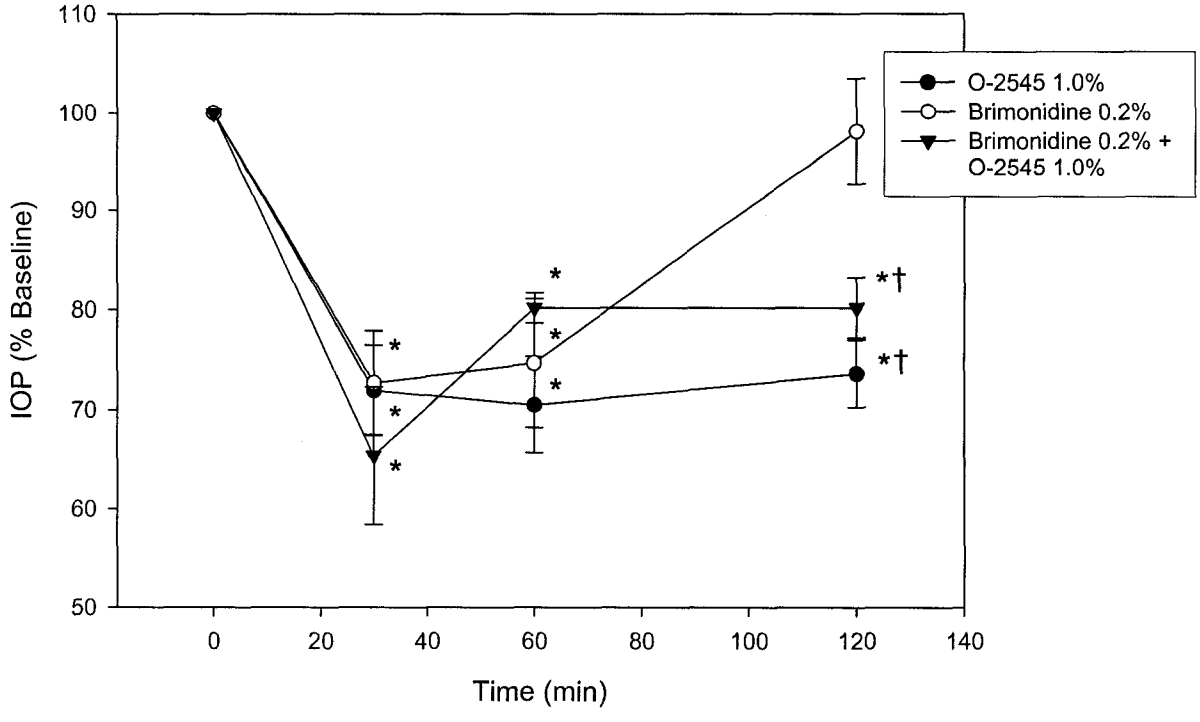

Figure 30: O-2545 1.0% and brimonidine 0.2% were applied alone or in combination and IOP recorded for 120 min.
Data are normalized to baseline measurements and presented as mean ± SEM.
* Significantly reduced compared to baseline (time = 0 min) ($p<0.05$)
† Significantly reduced than brimonidine ($p<0.05$)

Combination of O-2545 with brimonidine

Brimonidine and O-2545 were administered alone or in a fixed dose combination to evaluate their effect on IOP (figure 30). At the beginning of the experiment, baseline IOP measurements were obtained after which O-2545 1.0% or brimonidine 0.2% were applied (n=4 per group). For the combination treatment, brimonidine 0.2% was applied first followed 10 minutes later with O-1812 1.0% (n=4).

After 30 min, brimonidine reduced IOP from a baseline of 23±1.5 mmHg to 16±1.1 mmHg, O-2545 reduced IOP from a baseline of 25±1.4 mmHg to 18±0.4 mmHg while the combination treatment reduced IOP from a baseline of 27±0.9 mmHg to 17±1.3 mmHg (n=4). Although combination treatment reduced IOP beyond either drug alone, it was not significant.

In all treatment groups, maximal effect was achieved 30 min after drug administration, after which IOP reverted towards baseline measurements except for the O-2545 group. At the end of the experiment (120 min), IOP reduction was sustained at 18±0.5 mmHg in the O-2545 group and at 22±1.3 mmHg in the combination group. However, the brimonidine treatment reverted to 22±0.5 mmHg which was not significantly different from baseline (23±1.5 mmHg).

Effect of travoprost on IOP

Travoprost was administered alone and IOP monitored for 120 min. After 60 min, IOP significantly increased from a baseline of 20.3±0.7 mmHg to 23.5±0.6 mmHg (*p=0.009*, n=4). IOP returned to 20.5±0.6 mmHg after 120 min. Because of this variable response in IOP, further combination study was not performed.

Discussion

Although increased IOP is not the sole risk factor for development and progresion of glaucoma, therapies aimed at reducing IOP are beneficial for clinical management of glaucoma [35, 184]. It has been reported that even after a 1 mmHg reduction in IOP, the risk for glaucoma progression decreases by 10% [2]. With currently available drugs, almost 75% of patients required combination therapy after 2 years of monotherapy [185] to achieve a sufficient IOP reduction.

In this study, topical application of O-1812 and O-2545 was compared with timolol, a ß-adrenergic blocker and first line agent in glaucoma medical therapy [186-195]. The combined effect of O-1812 1.0% and timolol 0.5% or O-2545 1.0% and timolol 0.5% was significantly less from that of either drug alone suggesting that the combination did not produce additive or synergistic effects. Additionally, timolol made no significant contribution to the magnitude of the IOP reduction. It is therefore possible that the CB agonists may in some fashion draw on a receptor population that includes those affected by timolol. Vasquez et al have demonstrated that CB receptors can reduce beta

adrenergic receptor activity through G protein modulated effects, thus mimicking beta adrenergic blockade [196].

Dorzolamide is a carbonic anhydrase inhibitor. The exact mechanism of action of dorzolamide is unclear [197]. Vasodilation associated with dorzolamide is independent of pH changes in ocular vessels and activation of nitric oxide [198, 199]. Although, carbonic anhydrase inhibitors decrease capillary extracellular pH, the vasodilator effect might be potentiated by relaxation of capillary pericytes [200]. When administered alone, dorzolamide was as effective as O-1812 initially in reducing IOP but the duration of effect was short-lived. Dorzolamide was more effective than O-2545. When combined with either O-1812 or O-2545, no additive or synergistic effects were observed. In fact, the combination therapy had less on IOP than either drug alone. When combined with O-1812, IOP was sustained for up to 120 min, an effect similar to that of O-1812 alone. The sustainability of effect with the combination can be mostly attributed to O-1812, a potent CB1 agonist. In comparison, the duration was shorter with dorzolamide and O-2545. A suggestion for the stunted effect might be that since O-2545 is a mixed CB agonist, it might not be able to activate enough CB1 receptors. Although reports on this topic are scarce, Maor et al has suggested that cannabinoids do not inhibit carbonic anhydrase, this needs further study [201].

The α-adrenergic agonists initially reduce aqueous humor production [202-204] and increase uveoscleral outflow after chronic application [99]. In this rat glaucoma model, brimonidine alone was the least effective agent in terms of potency and duration of effect. When combined with O-1812, IOP was less than after brimonidine alone but was similar

to that after O-1812 alone. When combined with O-2545, the initial reduction in IOP appeared to be additive, but the duration of effect was short lived. Brimonidine lowers IOP by both reducing aqueous humor production and increasing uveoscleral outflow and therefore has the theoretical advantage over other medications. However, in numerous studies, brimonidine alone treatment was not as efficacious and not well tolerated [98]. Indeed, in our rat glaucoma model, brimonidine was the least effective agent especially when administered in combination with cannabinoids. In the presence of cannabinoids, the common G-proteins shared by both cannabinoid and α-adrenergic agonists might be redistributed [205].

Prostaglandin analogs are also emerging as conventional drugs for glaucoma management [206, 207]. In addition to its effect on IOP, it can also influence cyclo-oxygenase 2 and nitric oxide synthase to confer neuroprotection [208, 209]. Administration of travoprost in our rat glaucoma model actually increased IOP, and, therefore, was deemed unsuitable for the combination experiments. In other reports, prostaglandin analogs have been shown to elicit a bi-phasic IOP response in rodent models, i.e. an initial increase followed by a decline in IOP [210].

In this study, both O-1812 and O-2545 were most effacacious when administered alone. There were no synergistic or additive effects on IOP when combined with timolol, brimonidine or dorzolamide.

CHAPTER X

CONCLUSIONS

It has been over a decade since a new class of pharmacologic agents was introduced for the clinical management of glaucoma [25]. The currently available drugs to treat glaucoma primarily reduce the risk factor of elevated intraocular pressure. These drugs have not shown to be neuroprotective, which would address the underlying pathology present in glaucoma, as well as a number of other retinal degenerative disorders. That being said, it is true that therapies aimed at reducing IOP are beneficial for clinical management of glaucoma [35, 184]. Even a 1 mmHg reduction in IOP reduces the risk for glaucoma progression by 10% [2]. With currently available drugs, almost 75% of patients required combination therapy after 2 years to achieve a suitable IOP reduction [185]. This is an indication that there is a need for more efficacious drugs and for new therapuetic targets.

In this dissertation, newly synthesized cannabinoids, O-1812 and O-2545, were studied for their ability to decrease IOP and neuroprotective effects. The first aim with these new cannabinoids was to determine the pharmacokinetics after topical administration. Using *in vivo* microdialysis, continuous sampling of the aqueous and vitreous is possible to determine pharmacokinetic profiles of both the lipid (O-1812) and water soluble (O-2545) cannabinoids. After a single topical administration, O-1812 effectively penetrated both the anterior and posterior chamber and was maintained for at

least 120 min. O-2545, being water-soluble, also penetrated both the ocular chambers, but was eliminated more rapidly than O-1812.

Before studying the IOP effects in the eye, a stable ocular hypertensive model that mimics human glaucoma had to be developed. Limitations with currently available models prompted us to develop a technique in which careful attention was given to minimizing surgical trauma, primarily by identifying the vortex vessels directly through the conjunctiva and then ligating them using only very small minimal incisions. We hypothesized that the greater the surgical insult, the faster and more extensive the development of collateral circulation, which would defeat the attempt to elevate IOP. It was crucial that incisions of the conjunctiva be limited to the immediate area of the vortex veins. Ligation rather than cautery of vortex veins was essential. Collateral circulation was more extensive when cautery alone or cautery following ligation was used. It is possible that the use of cautery alone is a sufficient local insult to stimulate the formation of collaterals. Following ligation of three of the four vortex veins, a steady elevation was achieved without negative impact on other ocular structures except for the retina. After ligation, IOP increased gradually and was sustained for up to a year. This Sprague Dawley rat model was also remarkably free of postoperative complications and responded well to standard pharmacotherapy with timolol.

Using this ocular hypertensive model in specific aim # 2, the ability of newly synthesized O-1812 and O-2545 to decrease IOP was found to be comparable to or even greater than WIN 55212-2, a widely studied cannabinoid and timolol, a ß-adrenergic

blocker and first line agent in glaucoma medical therapy [186-195]. The ability of the cannabinoids to reduce IOP is mediated primarily by CB1 receptor activation. This can be concluded by using CB receptor specific antagonists. In the presence of CB1 antagonist, SR141716, the magnitude and duration of cannabinoid mediated hypotensive effect was severely diminished.

Once the potential to reduce IOP was realized, the neuroprotective effect of these O-compounds was studied in specific aim # 3. In this study the ERG was used as an end-point to evaluate retinal function based on the a-wave amplitude. The ERG a-wave represents the outer retinal response, which is generated by the photoreceptors and bipolar cells [89, 211]. The a-wave amplitude was preserved after treatment with both O-1812 and O-2545 when compared to NMDA alone treatment. However, O-1812 was more efficacious than O-2545. Retinas from a subsection of animals were removed and examined histologically. Our ERG findings corroborated with histology. Although both O-compounds were equally efficacious in preventing retinal ganglionic cell loss, data from both IOP as well as ERG suggests that among the O-compounds, O-1812 is more efficacious than O-2545.

In this study the electroretinogram was used as an end-point to evaluate retinal function based on the a-wave amplitudes. The ERG a-wave represents the outer retinal response which is generated by the photoreceptors and bipolar cells [89, 211]. The rods and cones with impulses from their respective bipolar cells contribute to the corneal positive and negative responses of the ERG a-wave [212]. However, majority of the a-wave

amplitude is derived from the rods rather than the cones [213]. The outer retinal response can be isolated by bright light stimulus [89, 211]. Although this technique is gaining popularity, analysis and interpretation of ERG data are currently not standardized. We are solely relying on the amplitude changes of the a-wave and not the latency of the peak largely due to the fact that the exact mechanisms that generate the a- or b-waves have not been fully elucidated. As a result, these findings cannot be translated directly in the human glaucoma condition. In this experiment, discrepancies in ERG a-wave response after NMDA damage were obvious within the first week. In some instances, photoreceptor damage did not register electrically with the full-field system and therefore was not used as part of the analysis. Fortunately, after two weeks, ERG responses had stabilized and differences between the treated and untreated eyes could be easily quantitated.

While this study solely focused on the health of the photoreceptors, the next logical step is to study the effects on the retinal ganglionic cells. The full-field ERG modality alone is not sufficient to assess ganglionic cell function, a more sophisticated ERG modality, such as a multifocal electroretinogram should be employed. With this technique, a functional map of the retina can be obtained *in vivo*. Regardless of the technological sophistication, the use of any ERG modality is still preferred over the conventional histological techniques because it is possible to monitor the progression of the disease as well as treatment effectiveness over time. A standard histological technique to count retinal cells involves an invasive procedure in which the optic nerve is transected and fluoro-gold injected at the transection site. The animal is then kept alive

for 48 hrs after which it is euthanized and the retinas flat mounted. This technique introduces a myriad of variables, for e.g. during the 48 hrs, the animal is rendered blind and loses the ability to feed itself, the dye itself is absorbed into the brain causing other CNS deficits. All of these side effects are not desirable.

Despite the currently available therapeutic approaches for the treatment of glaucoma, up to 50% of patients cannot be maintained on single drug therapy; most require use of two or even three drugs to control their IOP [97]. In additional experiments, the combined efficacy of cannabinoids with timolol, dorzolamide or brimonidine was compared in our rat glaucoma model. Travoprost was also used but did not change the IOP after a single dose. In this study, both O-1812 and O-2545 were most effacacious when administered alone. There were no synergistic or additive effects on IOP when combined with timolol, brimonidine or dorzolamide. Because of the efficacy of the O-compounds, they hold tremendous potential as monotherapy in patients who do not respond to currently available medications.

Glaucoma is a complex disease and its current limited therapeutic management suggests the need for neuroprotective intervention. While this neuroprotective strategy is sound, its realization requires dealing with complex signal transduction events that have unresolved mechanisms. However, data from this dissertation supports cannabinoids as a new class of drugs that have shown great potential for monotherapy, having both neuroprotective and anti-hypertensive effects. These initial observations need to be further confirmed in other animal models before they receive clinical evaluation. Future

investigations will focus on the determining the mechanisms of neuroprotection and development of new cannabinoid and non-cannabinoid analogs.

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Honors and Awards (*selected from a list of 12*)

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Patents

August 2006 – Initial application for a use patent filed with Eastern Virginia Medical School – Office of Technology Transfer for endocannabinoid analog, O-1812.

Presentations/Abstracts (*selected from a list of 27*)

Sandeep Samudre, Frank A Lattanzio, Jr., Billy R Martin, Ivan Castillo, Alireza Hosseini, Mauro Frazier, and Patricia B Williams, Ocular Pharmacokinetics and Pharmacodynamics of Synthetic Cannabinoid, O-1812, in a Perfused Rat Eye Model *FASEB J.* 21: 566.6

Publications (*selected from a list of 7*)

Oltmanns, M. and **Samudre, S.S.**, Castillo, I.G., Hosseini, A., Lichtman, A., Allen, R.C., Lattanzio, F.A., Williams, P.B. Topical WIN 55-212-2 Alleviates Intraocular Hypertension in Rats through a CB1 Receptor Mechanism of Action. *J Ocul Pharmacol Ther.* 2008;24:104-115

Research Grants

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